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RESEARCH ARTICLE

DIFFERENTIAL DIAGNOSIS OF MALARIA BY PERIPHERAL BLOOD SMEAR, ANTIGEN DETECTION AND CENTRIFUGED BUFFY COAT SMEAR EXAMINATION.

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Abstract

Purpose: Malaria continues to be a global public health challenge especially in the tropical and sub-tropical countries. Control and eradication of malaria have become very challenging issues and requires prompt treatment to save the patient's life, which in turn requires a rapid and accurate diagnosis. Several approaches have been developed in recent times to enable early and reliable diagnosis of malaria. Each of the techniques has their own advantages and disadvantages. The present study was undertaken to assess the usefulness of a modified centrifuged buffy coat smear (CBCS) technique for diagnosis of malaria and to compare it with a antigen detection test and conventional PBS examination. **Material and methods:** The present study was conducted on the blood samples of patients with suspected case of Malaria received in the department of Microbiology MMIMSR, Mullana. 50 positive samples were taken by RDT and then compared with PBF and CBC. The present study demonstrated the performance of a modified technique for diagnosis of malaria by incorporating a centrifugation-enhanced step into the conventional method of smear preparation and examination for malaria. This helps to concentrate the parasites, which are then easily visualized by microscopy and compared with rapid diagnostic test by antigen detection kit and conventional peripheral blood smear. **Results:** It was observed that while both PBS (100%) and CBCS (100%) had excellent specificity, But PBS had low sensitivity (94%) in detecting the malaria parasites as compared with CBCS (100%). **Conclusion:** It was concluded that CBCS is an easy, rapid and accurate technique and could be adopted for reliable diagnosis of malaria in resource-limited settings where RDT and QBC may prove to be costlier options.

Introduction:-

Malaria, sometimes called the ―King of Diseases, is caused by protozoan parasites of the genus Plasmodium.[1] Malaria is one of the most important infectious diseases in the world and its history extends into antiquity . Malaria or a disease resembling malaria has been noted for more than 4,000 years. From the Italian for "bad air," mal'aria has probably influenced to a great extent of human populations and human history. [2]

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Malaria imposes great socio-economic burden on humanity, and with six other diseases (diarrhea, HIV/AIDS, tuberculosis, measles, hepatitis B, and pneumonia), accounts for 85% of global infectious disease burden. [3]
A clinical diagnosis of malaria is traditional among medical doctors. Clinical diagnosis is based on the patients 'signs and symptoms, and on physical findings on examination. The earliest symptoms of malaria are very nonspecific and variable, and include fever, headache, weakness, myalgia, chills, dizziness, abdominal pain, diarrhea, nausea, vomiting, anorexia, and pruritus.[4]

Rapid and effective malaria diagnosis not only alleviates suffering, but also decreases community transmission. The nonspecific nature of the clinical signs and symptoms of malaria may result in over-treatment of malaria or non-treatment of other diseases in malaria-endemic areas, and miss diagnosis in non-endemic areas.[5]

In the laboratory, malaria is diagnosed using different techniques, e.g. conventional microscopic diagnosis by staining thin and thick peripheral blood smears, other concentration techniques, e.g. quantitative buffy coat (QBC) method, rapid diagnostic tests, and molecular diagnostic methods, such as polymerase chain reaction(PCR) . Some advantages and short comings of these methods have also been described, related to sensitivity, specificity, accuracy, precision, time consumed, cost-effectiveness, labor intensiveness, the need for skilled microbiologist, and the problem of inexperienced technicians.[6, 7]

New diagnostic methods and approaches are increasingly important in efforts to improve surveillance, the precision of transmission data, and the detection of cases of malaria infection. The ability to reliably diagnose malaria infection is fundamental to both the management of individual patients as well as public health efforts to control the disease. The working group on malaria and the UN Millennium Project took a number of measures into account in their proposed global plan, which involves the measurable target to reduce malaria morbidity and mortality by 75% by 2015 from the 2005 baseline level.[8]

However, asymptomatic carriers and patients with mild clinical manifestations and low parasitic density in less endemic countries are more common, and detecting infection in these people is more important because these asymptomatic carriers will continue to cause onward transmission silently.[9, 10]

Moreover, quality assurance protocols for microscopy are difficult to implement in elimination areas. Although microscopic diagnosis is sensitive and specific and remains the standard method for diagnosing malaria, it is not universally available, not considered a rapid diagnostic method, and requires a high level of expertise. Fluorescence microscopy was shown to improve the sensitivity, but not the specificity, of habitual microscopy-based procedures. Because of concerns about the sensitivity of rapid diagnostic tests in infections with low parasite densities, and their uncertain specificity for species other than P. falciparum, standardized quality assurance protocols are needed to confirm the diagnosis in the large numbers of suspicious negative results.[11, 12, and 13]

A clinical diagnosis of malaria is still challenging because of the non-specific nature of the signs and symptoms, which overlap considerably with other common, as well as potentially life-threatening diseases, e.g. common viral or bacterial infections, and other febrile illnesses. The overlapping of malaria symptoms with other tropical diseases impairs diagnostic specificity, which can promote the indiscriminate use of anti-malarial and compromise the quality of care for patients with non-malarial fevers in endemic areas.[14]

In the laboratory, malaria is diagnosed using different techniques, e.g. conventional microscopic diagnosis by staining thin and thick peripheral blood smears, other concentration techniques, e.g. quantitative buffy coat (QBC) method, rapid diagnostic tests, and molecular diagnostic methods, such as polymerase chain reaction (PCR). The accurate diagnosis of malaria infection is important in order to reduce severe complications and mortality. Malaria infection cannot be diagnosed clinically as the presenting clinical signs and symptoms mimic other tropical infections and therefore must be confirmed by laboratory diagnosis.[15]

A previous study from India had developed, standardized and reported on the feasibility of a modified centrifuged buffy coat smear (CBCS) examination for diagnosis of malaria in which a wintrobe's tube is used to obtain a buffy coat. This new technique combined most of the advantages of the existing techniques.

The main benefit of CBC is that they are more reliable and accurate to perform and interpret. Clinical diagnosis of malaria is extremely difficult even to an experienced medical practitioner. Reliable laboratory methods are needed to

assist the clinical diagnosis of malaria. Considering this, We conducted a study for the diagnosis of malaria with the aim to find out an easy, feasible and reasonable technique from the modifications of commonly employed techniques. The present study is based on **―comparative study of peripheral blood smear, antigen detection and centrifuged buffy coat smear examination for diagnosis of malaria"** In the present study, we have used the antigen tests as the gold standard.

The present study demonstrated the performance of a modified technique for diagnosis of malaria by incorporating a centrifugation-enhanced step into the conventional method of smear preparation and examination for malaria. This helps to concentrate the parasites, which are then easily visualized by microscopy. Keeping an eye on these state of the art techniques in the diagnosis of malaria, the present investigation was planned with clearly laid down ―Aims and Objectives‖ stated here after.

Material and Method:-

The present study was conducted on the suspected cases of Malaria whose samples were received from different wards and OPDs in the department of Microbiology MMIMSR, Mullana with a target of 50 positive cases by RDT. A relevant history has been taken from the patient and performa given in the Annexure I was filled. Clinical sample was blood. The study was carried out in the Department of Microbiology, Maharishi Markendeshwar Institute of Medical Science And Research for a period of one year from April 2015 to February 2016. A prospective study were carried out in the department of microbiology, MMIMSR, Mullana Study were conducted on 50 positive blood samples by RDT attending OPD and IPD of MMIMSR Mullana.

Sample Processing:-
Peripheral blood smear:-
Preparation of thick and thin smear:-
Thick smear: A big drop of blood is spread over half inch square area on a clean glass slide. The Thickness of the film should be such that it allow newsprint to be read. The film was dried and kept in distilled water in a koplin jar for 5-10 minutes for dehemoglobinization. **Thin smear:** A small drop of blood is taken on a corner of a slide. It is spread by another slide at an angle of 45oC and then is lowered to an angle of 30oC and is pushed gently to the left, till the blood is exhausted.

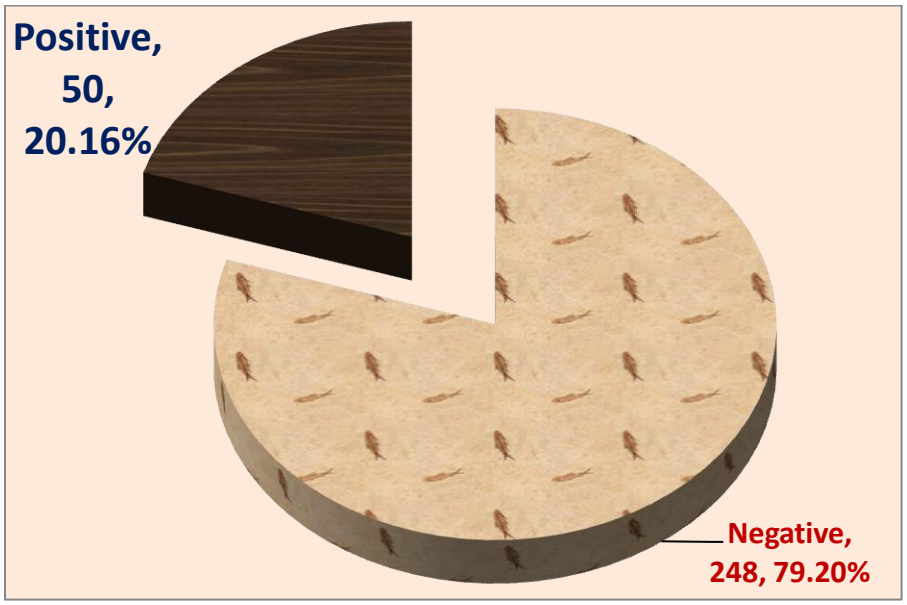
Centrifuged buffy coat smears were prepared by using 2 ml blood collected in a wide bore 4 ml tube with EDTA which was centrifuged (2000– 3000 rpm for 15 min). The supernatant plasma was separated and layer of buffy coat and equal thickness of RBC layer just below was picked up to prepare smears which were stained by leishman stain
Microscopic examination of the stained film.

Examine the blood smear first:-
Screen the smear near the feathery tail end. Screen 200-300 oil immersion fields examined before the smears are considered as negative then we screened thick smear. Interpretation of Results: Plasmodium vivax: detection of asexual forms i.e. ring form, late trophozoites and schizonts, gametocytes Plasmodium falciparum: gametocytes and ring forms.

Detection of Antigen:-
Antigen was detected by immune chromatographic (ICT) method.
Principle of the test: Utilized the principle of immune-chromatography. As the test sample flows through the membrane assembly of the device after addition of the clearing buffer, the colored colloidal gold conjugates of monoclonal anti Pf HRP-2 (IgG) antibody ad monoclonal anti *P.vivax*specific pLDH antibody complex the HRP-2-pLDH in the lysed sample. This complex moves further on the membrane to the test region where it is immobilized by the anti-Pan specific pLDH (monoclonal) antibody and /or the monoclonal anti Pf . HRP-2(Ig M) antibody coated on the membrane leading to formation of pink purple colored band/s which confirms a positive test result. A band appeared under Pf at the test region in falciparum positive samples, while a band will appear under Pv in vivax malaria positive samples. Appearance of band under Pf as well as Pv in the region suggested a mixed infection.

Result:-

A total of 248 clinically suspected cases of malaria were enrolled in the study. Out of 248 samples 50 blood samples were positive for malaria either by PBS or by RDT. Then on these 50 samples we have done comparison of PBS and centrifuged buffy coat smear examination by taking antigen detection test as gold standard.

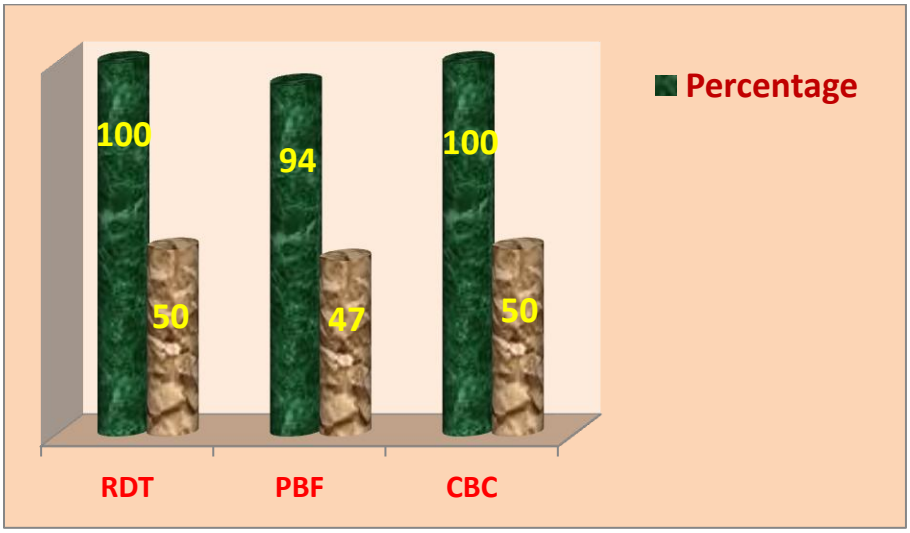


Graph 1:- Prevalence of malaria among clinically suspected cases.

Table 1shows the prevalence of malaria among clinically suspected cases. A total of 129 cases were found to be positive using rapid diagnostic test. Thus prevalence of malaria among clinically suspected cases was 20.15 %.

Table 1:- Prevalence of malaria among clinically suspected cases.

S. No.	Outcome	No. of Cases	Percentage (%)
1.	Test Negative	198	79.20
2.	Test Positive	50	20.16
3.	Total	248	100

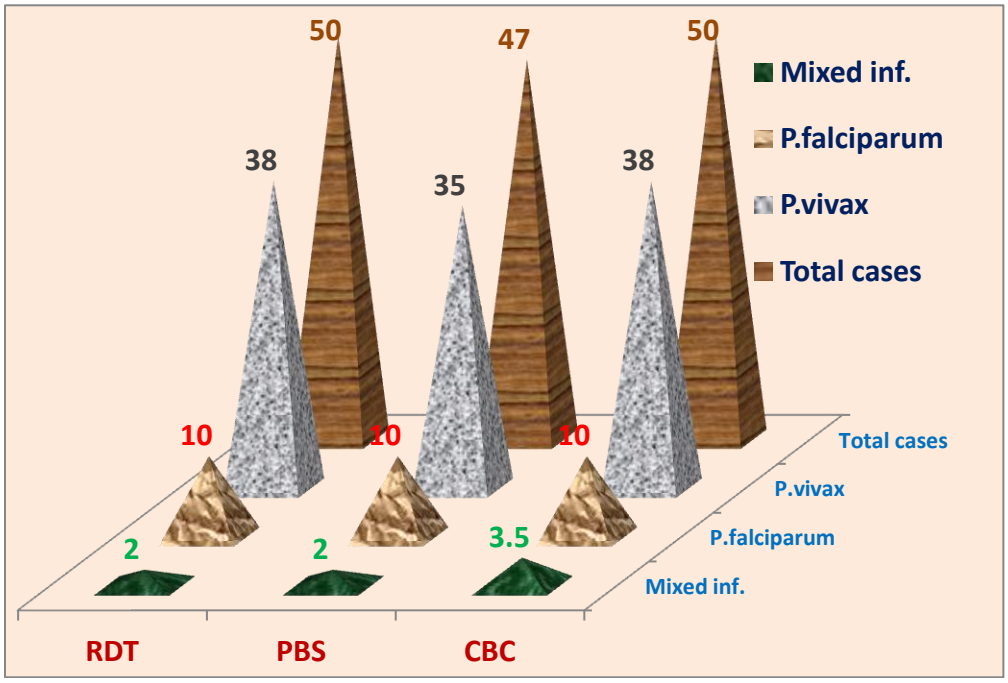


Graph 2:- Distribution of malaria positive cases using different methods.

Table 2 shows that during the study period, a total of 50 positive samples RDT from the patients were received for testing for malaria parasites. The three diagnostic modalities gave varied results. shows distribution of malaria positive cases using different methods. Percentage positivity of three techniques ranged from 94.00% [PBF] to 100% [RDT AND CBC].

Table 2:- Distribution of malaria positive cases using different Methods

S. No.	Method	Test Positive	Percentage %
1	RDT	50	100
2	PBF	47	94
3	CBC	50	100

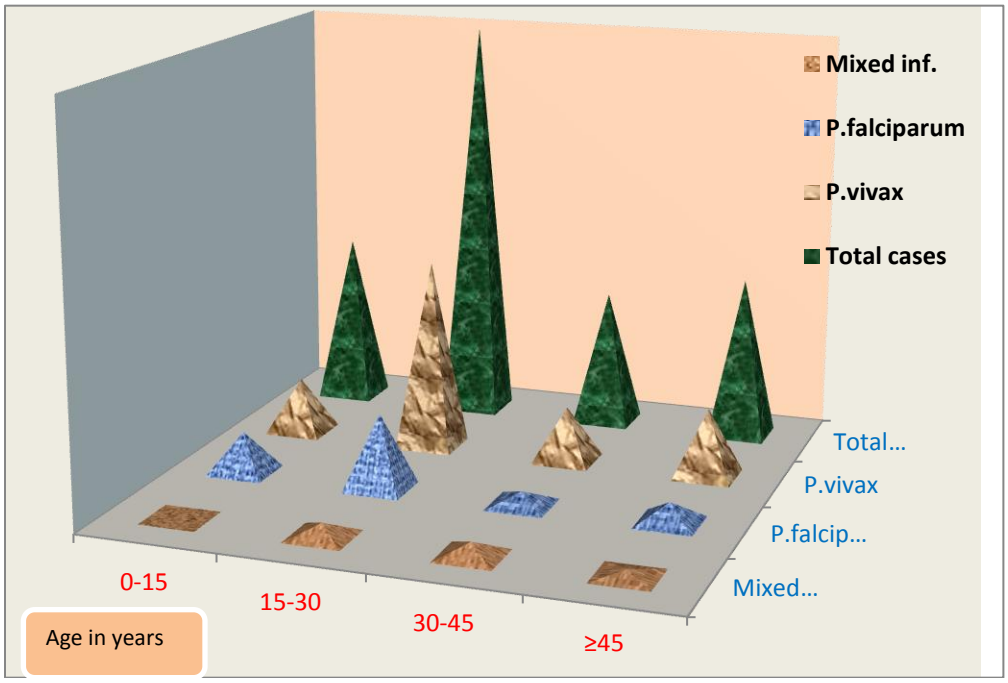


Graph 3:- Species distribution of malaria parasites in three different methods. (*n*=50)

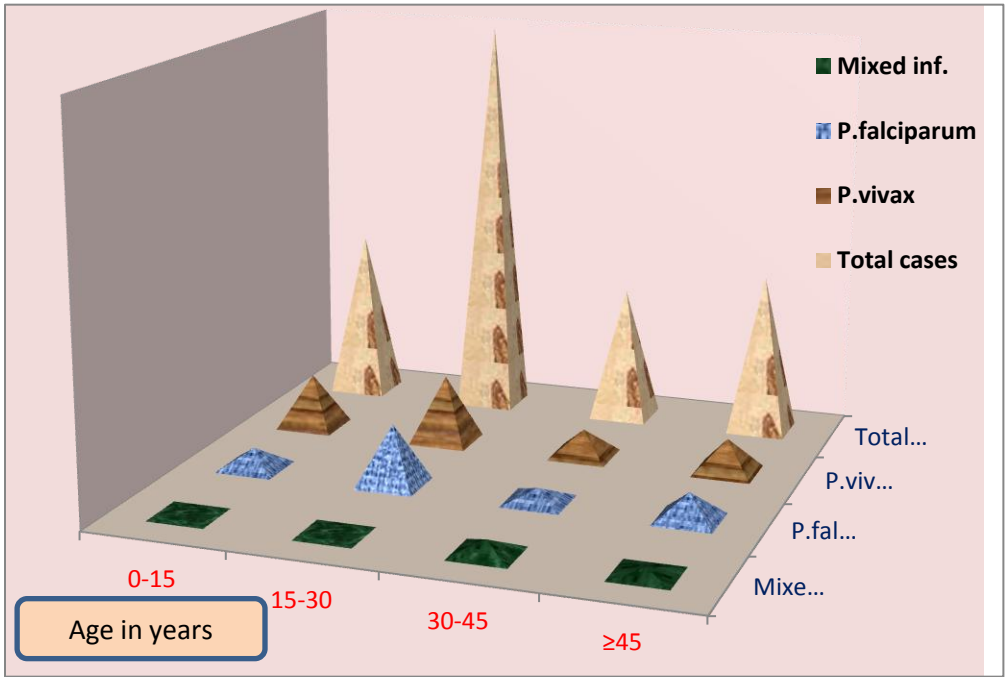
Table 3 shows the total number of malaria positive cases was found to be 50. All the three methods detected P.vivax, P.falciparum and mixed infection. Using RDT. P. vivax[38], P. falciparum [10] and mixed infection [02] out of total 50 blood samples. By preparing PBS P.vivax[35], P.falciparum[10] and Mixed infection [02] out of total 50 blood samples. And by CBC P.vivax [38], P.falciparum [10] and mixed infection 2 out of total 50 blood samples.

Table 3:- Occurrence of different malarial species using three different methods.

SPECIES	RDT n-50	PBS n-50	CBC n-50
P.vivax	Positive-38	Positive-35	Positive-38
P. falciparum	Positive-10	Positive-10	Positive-10
Mixed Infection (P vivax.+ P. falciparum)	Positive-02	Positive-02	Positive-02
Total	50	47	50



Graph 4 (a) :- Age and (male) Gender wise distribution of malaria.

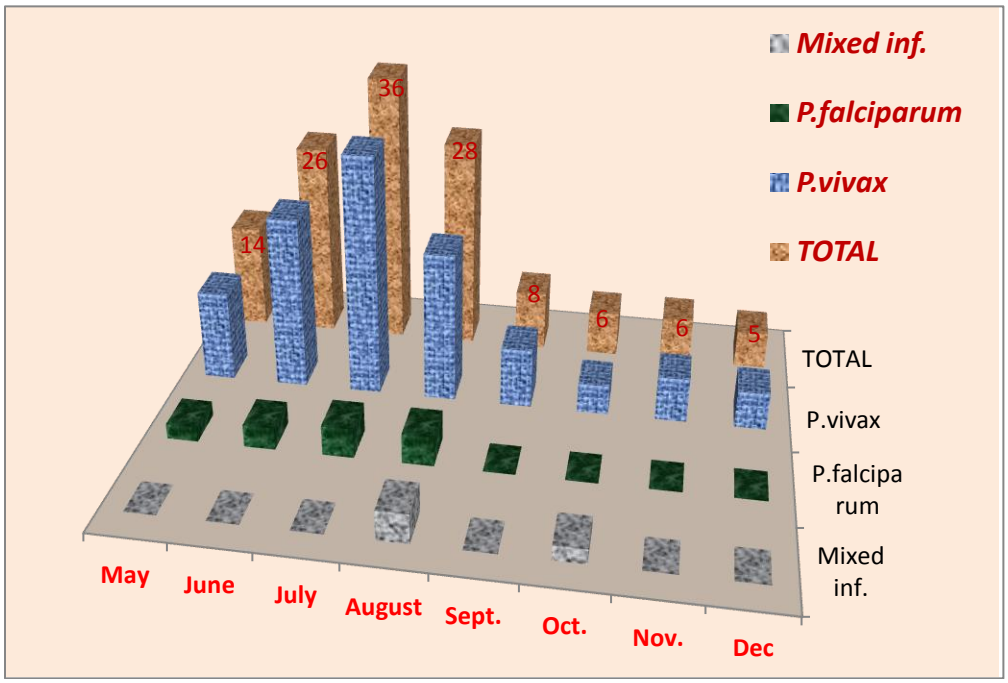


Graph 4 (b) :- Age and (female) Gender wise distribution of malaria..

Table 4 shows age wise and gender distribution of malaria. . The maximum number of cases were in the age group of 15-30 years (46%), while the minimum number of cases were in the age group of >45 years (12%). The youngest patient in this study was 2 year old while the oldest was 86 years old. Also shows distribution of malaria positive patients according to gender. In the present study, a total of 81(62.7%) patients were male and remaining 48(37.2%) were female. The male to female ratio of the malaria positive subjects was 1.7:1.

Table 4:- Shows the age wise and gender wise distribution of malaria.

Age In year.	Total	MALE			FEMALE		
		P.vivax	*P.falciparum*	Mixed Infection	*P.vivax.*	*P.falciparum.*	Mixed Infection
0-15	11	5	3	0	2	1	0
15-30	17	4	3	2	4	2	2
30-45	12	6	1	1	2	2	0
>45	10	5	2	1	1	0	1
Total	50	20	9	4	9	5	3

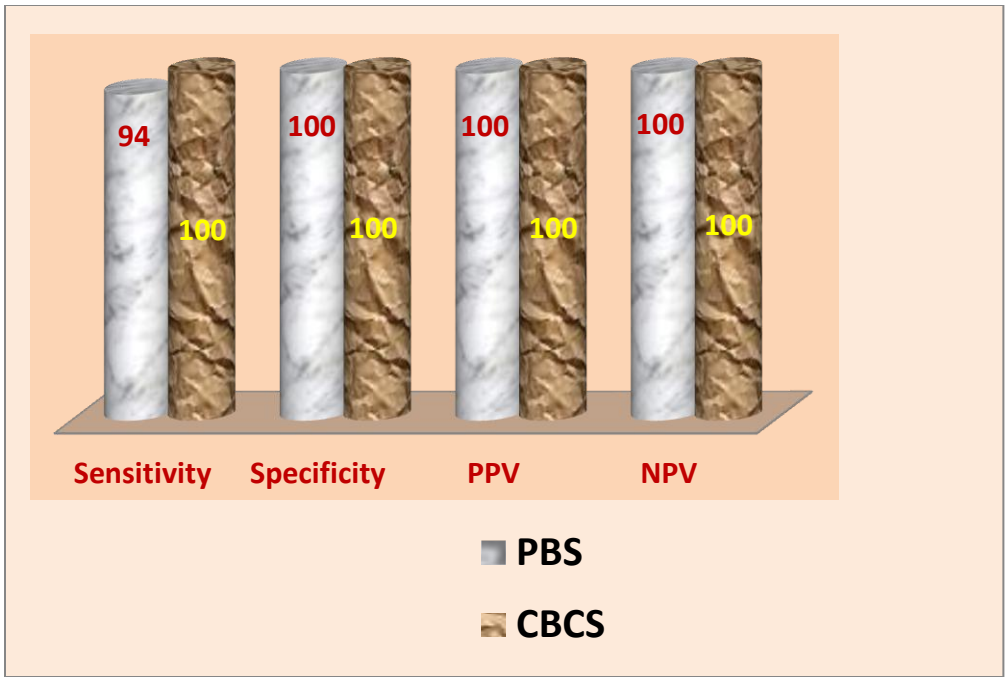


Graph 5:- Month wise distribution of malarial species.

Table 5 Shows the distribution of malaria positive patients according to change in season. A maximum of 11 and 13 were seen in the month of July and August and minimum 2 and 2 were seen in the month of November and December.

Table 5:- Month wise distribution of malarial species.

Month	Total	*P.vivax*	*P. falciparum*	Mixed Infection
May	5	3	02	0
June	9	6	03	0
July	11	7	04	0
August	13	5	04	4
September	3	3	0	0
October	5	3	0	2
November	2	2	0	0
December	2	2	0	0
Total	50	31	13	6



Graph 6:- Sensitivity, specificity, positive predictive value, negative predictive value of PBF and CBCS in comparison to antigen test.

Table 6 shows the specificity, sensitivity, of PBS and CBCS in comparison to antigen test. It was observed that while both PBS (100%) and CBCS (100%) had excellent specificity, PBS had low sensitivity (94%) in detecting the malaria parasites as compared with CBCS (100%).

Table 6:- Sensitivity, specificity, positive predictive value, negative predictive value of PBF and CBCS in comparison to antigen test.

TEST	SENSITIVITY	SPECIFICITY	PPV	NPV
PBS	94%	100%	100%	100%
CBCS	100%	100%	100%	100%

Discussion:-

Half of the world's population is at risk of malaria, with an estimated 243 million cases worldwide. Prompt parasitological confirmation by microscopy or alternatively by RDTs is recommended for all patients with suspected malaria before treatment is started.[16]

The results of present study indicated that 50 (20.16%) were infected with malaria and and the rest 198 (79.20%) were malaria negative out of total 248 blood samples. [TABLE-1]

This matched the results of the study done by **Iqbal et al** [17] [2003] who obtained blood specimens from 930 suspected malaria patients attending BHU and found 231 [25%] to be positive.

From the above finding, it is observed that some results are closed to and some remarkably differ from the present study. The difference may be due to the fact that studied were conducted at different geographical areas and the disease prevalence differ from region to region. **Aggarwal N et al** [18][1997] concluded that the event of positivity might depend on the relative prevalence of malaria in different regions. Although according to World Health Organization Malaria risk chart [2011], major part of India falls into malaria risk zone.

In the present study it shows the distribution of malaria positive cases using different methods. Percentage positivity of three techniques ranged from 94.00% [PBF] to 100% [RDT AND CBC].[TABLE-2]

The result was comparable with the study done by **S Mohanty et al.**[19] [2015] . Of 1655 samples received, 394 (23.8%) samples were positive for infection with malaria parasites. All the three tests detected malaria infection

equally in 279 samples, and gave varied results in the remaining 115 samples. Addition of centrifugation (i.e. CBCS) to the conventional method of PBS enabled detection of 80 more cases of plasmodia infection, especially (43, 53.7%) at low levels of parasitemia (<200 parasites/µl). While both PBS and CBCS had excellent specificity (99.7% and 99.2%, respectively), PBS examination had low sensitivity (72.9%) in detecting malaria parasites in comparison to CBCS. The sensitivity of CBCS in detecting malaria parasites was 91.9%.

Our examination by three methods detected P.vivax, P.falciparum and mixed infection. Using RDT. P. vivax [38], P. falciparum [10] and mixed infection [02] out of total 50 blood samples. By preparing PBS P.vivax [35], P.falciparum [10] and Mixed infection [02] out of total 50 blood samles. And by CBC P.vivax [38], P.falciparum [10] and mixed infection 2 out of total 50 blood samples. [TABLE:3]

Result of our study matched with those of **S Mohanty et al** [19][2015] who tested species distribution of malaria parasites detected by the different methods. In PBS, out of 283 samples, 115 were positive for *P. falciparum*, 165 for *P. vivax* and 3 samples were positive for mixed infection. The CBCS showed 363 positive for malaria of which 161 were *P. falciparum*, 198 were *P. vivax* and 4 were mixed infection. The antigen test was positive in 384 cases with 174 being positive for *P. falciparum*, 204 for *P. vivax* and 6 for mixed infection.

In the present study maximum number of cases falls in the age group of 15-30 years, while the minimum number of cases falls in the age group of both more <45 year and >16 year. The maximum number of cases were in the age group of 15-30 years (46%), while the minimum number of cases were in the age group of >45 years (12%). The youngest patient in this study was 2 year old while the oldest was 86 years old.[TABLE 4]

Similar study was done by **Ashwani Kumar et al** [20][2007]. Most of the point prevalence studies in India have been carried out for outbreak/epidemic investigations. There is very limited information on age- and sex-specific seasonal prevalence of malaria in different paradigms in the country. In the available studies, age and sex classification used is arbitrary. The burden is generally higher in men than women in all age groups. Children in the states of Assam, Arunachal Pradesh, and Rajasthan had a higher incidence of malaria than adults, whereas in the indo gangatic plains, the situation was reversed.

The result of present study indicated that maximum of 36 (27.9%) and 28 (21.7%) were seen in the month of July and August and minimum 6 (4.6%) and 5 (3.8%) were seen in the month of November Table [5].This matched the results of the study done by **Idris M Z et al** [21] (2014) conducted two cross-sectional surveys during the dry July , August and two consecutive November and December. (31.1%) were higher in the July and august than in the (5.3%) in November and December.

Variation in malaria prevalence reflects the different dynamic of malaria transmission among different season. Our results provide baseline data for the planned feasibility study of malaria elimination. A diagnosis of malaria based on clinical grounds alone is therefore unreliable and, when In the present study it shows the specificity, sensitivity of PBS and CBCS in comparison to antigen test. It was observed that while both PBS (100%) and CBCS (100%) had excellent specificity, PBS had low sensitivity (94%) in detecting the malaria parasites as compared with CBCS (100%. predictive value, negative predictive value .[TABL 6]

Similarly the study done by **Mohanty S et al** [8][2015] which shows the specificity, sensitivity, positive predictive value and negative predictive value of PBS and CBCS in comparison to antigen test. It was observed that while both PBS and CBCS had excellent specificity, PBS had low sensitivity (72.9%) in detecting the malaria parasites as compared with CBCS (91.9%).

The three slides reported as negative out of fifty by peripheral blood smear, but on other hand these false negative slides were observed positive by centrifuged buffy coat smear and antigen detection kit .Thus performance of a modified technique for diagnosis of malaria by incorporating a centrifugation-enhanced step into the conventional method of smear preparation and examination for malaria which gives positive result even in the case of low parasitemia as compared with conventional peripheral blood smear examination which gives false negative result in low parasitemia.

Conclusion:-

The present study was done on the comparison of PBS, antigen detection test and CBC. As we know microscopic examination of peripheral blood smears (PBSs) as stained thick and/or thin blood smears is the standard method for malaria diagnosis, which is easily available and has low cost but its reliability is questionable at low level of parasitaemia and RDTs which are capable to detect malaria cases with good sensitivity and specificity but they are prove to be costlier options in resource limited areas. So the development of easy, rapid and accurate tests for the reliable detection of plasmodia infection is highly desirable. The present study ultimately concluded the performance of a modified technique for diagnosis of malaria by incorporating a centrifugation-enhanced step into the conventional method of smear preparation and examination for malaria which gives positive result even in the case of low parasitemia as compared with conventional peripheral blood smear examination which gives false negative result in low parasitemia. This helps to concentrate the parasites, which are then easily visualized by microscopy. The CBCS technique fulfills most of these criteria and may be adopted for rapid and reliable diagnosis of malaria in resource-limited settings.

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