Title: Comparison of different diagnostic techniques for detection of Malaria infection in blood samples collected from Malaria endemic areas of Assam and Arunachal Pradesh

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Abstract:

Background: According to World Health Organization, about 260 million people worldwide are infected with malaria and 1.5-2.7 million patients die annually. It is also a major problem in India. Prompt and accurate diagnosis of malaria is the key to prevent disease morbidity and mortality.

Design of the study: Blood samples were collected from 188 individuals having history of malaria/suspected with malaria during period from August, 2011 to July, 2012 from different block PHC under different malaria endemic area of Assam and Arunachal Pradesh.This investigation was conducted to compare the diagnostic performance of five routinely used methods viz, Microscopic slide experiment, ParaHit kit, SD Bioline malaria Antigen test, Advantage Mal Card test kit and Polymerase chain reaction (PCR) methods (assuming PCR as standard method) for detection of malaria parasite.

Results: PCR (gold standard method) results showed that 21.28% of the total cases were positive for *Pf*, 0.53% positive for *Pv* and 3.72% for mixed infection. In comparison with PCR, Microscopic slide examination was 81.25% sensitive and 95.83% specific for detection of malaria parasite. The sensitivity and specificity of SD Bioline malaria antigen test was 95.83% and 85.42% respectively whereas Advantage Mal card test for detection of malaria parasitaemia gives 91.67% sensitivity and 85.42% specificity. However, ParaHit-f kit test exhibited lowest sensitivity (77.08%) and specificity (68.75%) among the five diagnostic methods.

Conclusion: From the above mentioned result we conclude that ParaHit-f kit test appears to be poor sensitive and less specific for detecting malaria parasites. Although PCR cannot be done in field, considering its high degree of sensitivity and specificity, it can be used for further confirmation of malaria.

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1. Introduction:

Malaria is a mosquito-borne infectious disease of humans and other animals which becomes a devastating global public health problem. It is a major killer of mankind, especially in developing countries where it becomes one of the major causes of morbidity and mortality [1]. The disease affects the populations of tropical and subtropical areas worldwide. Among the 5 species of *Plasmodium* (*P. falciparum, P. vivax, P.ovale, P.malariae and P knowlesi*)that cause malaria in humans, *P. falciparum(Pf)* is the most dangerous and responsible for most of the morbidity and mortality[2]. According to the *World malaria report* 2011, there were about 216 million cases of malaria and an estimated 6, 55, 000 deaths in 2010.In India, 80% of total population (1.2 billion) lives in malaria risk areas [3].Officially, at least 1.5 million malaria cases are reported every year in India and they are divided evenly between *P. falciparum* (*Pf*) and *P. vivax*(*Pv*)[4, 5, 6].

Irrespective of an major public health problem, lack of prompt and sensitive diagnosis continue to be an major drawback in controlling malaria morbidity and mortality by effective and timely intervention of drugs in malaria endemic areas[7].In developing countries the traditional malaria diagnosis is based on the examination of stained blood smears under light microscope. Although, this method remains the gold standard for malaria diagnosis however microscopic examination is laborious and requires considerable expertise for its interpretation, particularly at low levels of parasitaemia [8]. Moreover, sometimes the parasites could be sequestered and are not present in peripheral blood of patients with *Pf* malaria. Thus, *Pf infection* could be missed due to absence of the parasite in the peripheral blood film. Besides these, majority of malaria cases occur in rural areas where there is little or no access to reference laboratories and moreover microscopy is not available in many areas. However, it is labor-intensive, time-consuming, and more importantly, requires skills and experienced microscopists.

Recently, alternative methods, such as immunochromatographic assay, molecular amplification method, fluorescence microscopy, mass spectrometry, and flow cytometry have been developed for malaria diagnosis [9]. These methods have some advantages as well as few limitations on one another. Among different assays immunochromatographic assays are gaining importance especially in developing world where far flung remote areas do not have that much suitable infrastructure to meet the requirements for newer sensitive assays. Although the Enzyme linked immunosorbent (ELISA) assay for Malaria diagnosis is routinely used in different

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government as well as private hospital in India with a sensitivity of 98.15% and sensitiveof96%. However with availability of different ELISA kits, which are based on different principles it is sometime confusing for public health workers. Therefore it is important to asses them for sensitivity and specificity of these kits and also a comparative evaluation between these kits. There were no systemic studies carried out in malaria endemic areas of India to evaluate diagnostic efficacy of these kits. Therefore we undertake this study in Assam and Arunachal Pradesh, two north eastern state of India with high malaria endemicity to compare the diagnostic performance of routinely used kits and conventional method by state public health workers and private hospitals. Both Assam (89° *42′ E to 96° E* longitude *and 24° 8′ N to 28° 2′ N)* and Arunachal Pradesh *(*26° 30' N and 29° 30 ' N and *longitude* 91° 30' E and 97° 30'E) have recently shown increase in resistance to parasites against anti-malarial drugs, therefore it is an urgency or atmost importance to have the best diagnostic module in the public health sector for Malaria diagnosis.

2. Material and Methods:

2.1 Sample collection:

Two milliliter of blood samples were collected from 188 individuals (aged from range 1.6 to 70 years) having history of malaria/suspected with malaria. The study was carried out during period from August, 2011 to July, 2012 through household visit as well as from different Primary Health Centre (PHC) under different malaria endemic districts of Assam (Dibrugarh, Tinsukia, Karbi-Anglong and Chirang) and Arunachal Pradesh (Lohit, Changlang).Informed consent was taken from all the subjects before collecting the blood. Clinical history and drugs taken, if any, were recorded for each subjects. Institutional ethical clearance was obtained from institutional ethical committee, RMRC, ICMR, Dibrugarh, Assam

2.2 Malaria diagnosis Methods:

The study was performed by comparing five diagnostic methods viz. Microscopic examination, ParaHit-*f* Kit test, SD Bioline Malaria Antigen *Pf/Pan* Test, Advantage Mal Card test and PCR to compare the diagnostic performance for detection of malaria parasite in patient’s samples.

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2.2.1 Microscopic examinations:

Thick and thin blood smears were prepared for all blood samples and stained with 10% Giemsa. The malaria parasite was detected under light microscopy. Samples were considered negative when no parasite was detected after examining 100 microscopic fields.

2.2.2 ParaHit-*f* Kit test (Rapid diagnostic tests (RDTs) kit):

Malaria diagnosis by commercial RDT (Malaria Antigen Pf/PanTM) was performed in all blood samples (finger-prick blood) using the method as per standard protocol (http: //standardia.com/html_e/mn03/mn03_01.asp). This test utilizes the detection of histidine rich Protein II which is species specific test for *Pf* malaria [1]. The presence of both the control and test lines indicated a positive result for *P. falciparum*, whereas the presence of only the control line indicated a negative result. All RDT kits were stored as directed by the manufacturer and the quality of package desiccant was checked before use.

2.2.3 SD Bioline Malaria Ag*Pf/Pv* test:

One step malaria Ag rapid *Pf / Pv* test (SD Bio Standard Diagnostics)is recently introduced malaria diagnostic test contains a membrane strip, which is pre-coated with recombinant malaria *Pf* capture antigen (MSP) on test band 1 region and with recombinant malaria *Pv* capture antigen (MSP) on test band 2 region. This test device has a letter of 1, 2 and C as "Test Line 1", "Test Line 2" and "Control Line" on the surface of the case. The test was done as per the manufacturer instruction.

2.2.4 SD Bioline Malaria Antigen *Pf/Pan* Test:

The SD Bioline Malaria Antigen *Pf/Pan* Test (company-SD Bio Standard Diagnostics) is pre-coated with one monoclonal antibody (specific to the histidine rich protein II of *Pf*) and one polyclonal antibody (specific to the lactate dehydrogenase of *Plasmodium* species like *Pf, Pv, P. malariae, P. ovale*) as two separate lines across a test strip and the test was carried out as per the procedure provided in the kit.

2.2.5 Advantage Mal Card

Advantage Mal card (Company-J Mitra) evaluated by WHO is a visual, rapid and sensitive immunoassay for the qualitative diagnosis of infection with *Pf* and other *Plasmodium* Species (*Pf/ Pv/ P. malariae/ P.ovale*) in human whole blood. The technique is mainly based on Sandwich principle and the test uses monoclonal anti *Pf*pLDH antibody (test line F) and monoclonal anti-Pan specific pLDH antibody (test line P)as per manufacturer instruction.

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2.2.6 SD Bioline Malaria *Pf/Pv* test:

SD BIOLINE Malaria *Pf/Pv*(One Step Malaria P.f/P.v Antibody Test (company-SD Bio Standard Diagnostics) is a qualitative immunochromatographic rapid test for detection of antibodies of all isotypes(IgG, IgM, IgA) specific to *Pf* and *Pv* simultaneously in human serum, plasma or whole blood.

2.2.7 Detection of malaria parasite by PCR:

The PCR analysis for malaria was done in all the collected samples. DNA extraction from EDTA blood samples was done using the QIAamp DNA Mini spin columns kit (Millipore Corporation). The extracted DNA was thereafter amplified by the PCR using a set of primers as described by Snounou et al., 1996. In the first round of amplification a universal primer set was used to detect the malaria parasite whereas in the second step a set of species specific primer pairs was used for detection of specific parasite. The thermo-cycler was programmed as follows. Hot start- 95 °C for 5 min; initial denaturation-94 °C for 1 min; annealing -58°C for 2 min; extension-72 °C for 2 min; final extension 72 for 5 min. A total of 26 cycles were used and final annealing and extension was for 5 min. after which the reaction was stopped and the amplified product was detected by electrophoresis on 1.5% agarose gel containing ethidium bromide and visualized on an ultraviolet transillumination. The Gel photographs were taken and specific amplified bands at 50 bp were looked.

Statistical analysis

Simple linear regression was done using age trend as an independent variable and no. of confirm malaria cases as a dependent variable. Whereas Sensitivity, Specificity, Positive predictive value, Negative predictive value, false positive rate, false negative rate, Likelihood ratio positive and likelihood ratio negative of ParaHit-*f* Kit test , SD Bioline Malaria Antigen *Pf/Pan* Test , Advantage Mal Card and Microscopic examinations was calculated to know the accurateness diagnostic performance of these methods.

3. Results:

Among the 188 study subjects, a total of103 (54.79%) were males and 85 (45.21%) were females. The age of the subjects ranged between 1.6 to over 70 years (32.1 ± 15.29).Males (52.08%) were found to be highly prone to malaria than the female (47.92%). Most of the malaria cases were reported from Karbi Anglong district (37 nos) proceeded by Chirang (7 nos), Dibrugarh (1 no.), Tinsukia (1 no.), Changlang (1 no.) and Lohit (1no.) (Fig 5). Age wise

distribution analysis among the study subjects revealed that decreased in trend of malaria cases, with increasing age (Fig 6). All the suspected malaria patients were examined by thin and thick blood film analysis using light microscopy which revealed19.15%were having only *P. falciparum* and 2.67% as mixed infections (*Pf+Pv*).PCR (gold standard method) results showed that 21.28% of the total cases were positive for *Pf*, 0.53% positive for *Pv* and 3.72% for mixed infection. In comparison with PCR, Microscopic slide examination was 81.25% sensitive and 95.83% specific for detection of malaria parasite (Table 3), The sensitivity and specificity of SD Bioline malaria antigen test was 95.83% and 85.42% respectively (Table 3, Fig 2) whereas Advantage Mal card test (Fig 3) for detection of malaria parasitaemia gives 91.67% sensitivity and 85.42% specificity. However, ParaHit kit test exhibited lowest sensitivity (77.08%) and specificity (68.75%) among the five diagnostic methods (Fig1), by showing positivity of 27.67% (52/188) for *P. falciparum.* ParaHit Kit test shows highest false positive rate (31.25%) proceeded by SD Bioline malaria antigen P.f/Pan test (14.58%) , Mal Card test (14.58%) and Microscopic slide examination (4.17%) (Table 3).Whereas SD Bioline Malaria Ag*Pf/Pan* test gives lowest false negative result (4.17%) followed by Advantage Mal card Ag test (8.33%), Microscopic slide experiment (18.75%) and ParaHit-*f* Kit test(22.92%).

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Table 1: Personal characteristics of 188 suspected cases of malaria.

Age range	Nos. of cases	
	Suspected (n=188 nos)	**Confirmed in percentage (n=48 nos)**
Below 10	35	17 (48.57)
10 to 19	33	10 (30.30)
20 to 29	43	10 (23.26)
30 to 39	34	04 (11.76)
40 to 49	20	04 (20)
50 to 59	15	03 (20)
60 to 69	07	
70 to 79	01	
Total	**188**	**48**

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Table 2: Result showing the comparative performance of different diagnostic methods

Result	Percentage (%)				
	Microscopic slide	ParaHit-*f* kit	SD-Bioline Malaria Ag *Pf/Pan*	Advantage Mal card	PCR
Negative	78.19	72.34	71.81	72.34	74.47
Pf	19.15	27.67	25.00	24.47	21.28
Pv	------	-------	------	-------	0.53
Mixed	2.67	------	3.19	3.19	3.72

Table 3:The test performance of different diagnostic method for detection of *P. falciparum* and *P. v compared* with the PCR method:

Test performance of different kits	Percentage (%)			
	Microscopic slide	ParaHit-*f* Kit test	SD Bioline Malaria Ag *Pf/Pan* test	Advantage Mal Card Ag test
Sensitivity	81.25	77.08	95.83	91.67
Specificity	95.83	68.75	85.42	85.42
Positive predictive value	95.12	71.15	86.79	86.54
Negative predictive value	83.64	75	95.35	93.18
False positive rate (α)	4.17	31.25	14.58	14.58
False negative value (β)	18.75	22.92	4.17	8.33
Likelihood ratio positive	19.48	2.47	6.57	6.29
Likelihood ratio negative	0.20	0.34	0.049	0.098

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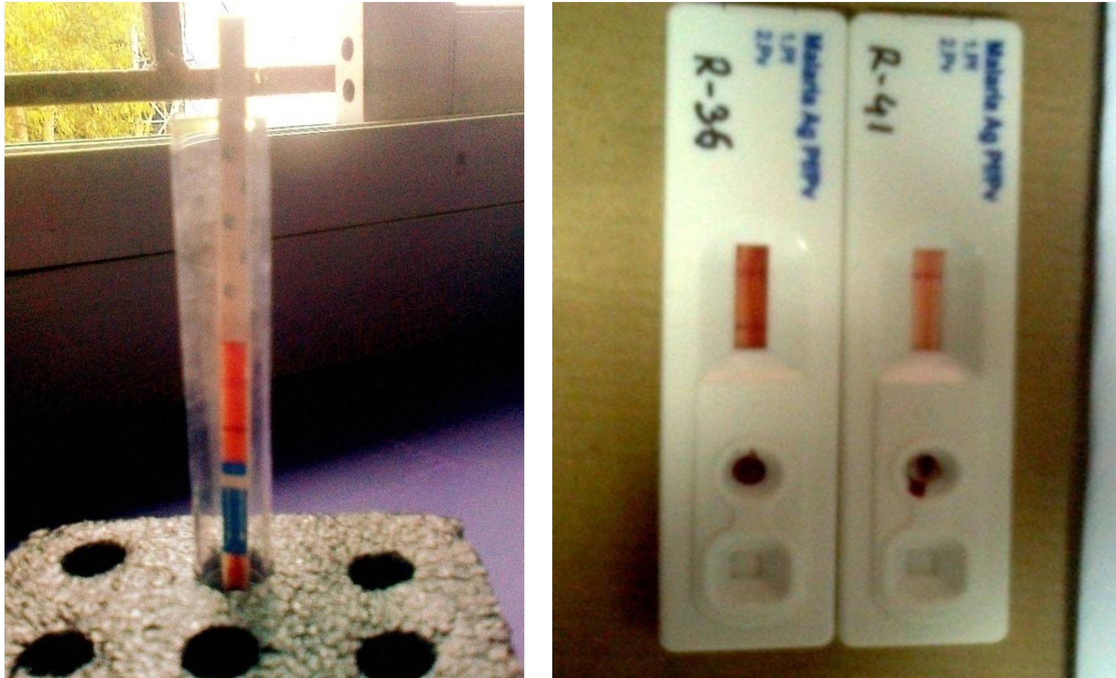

Fig 1: ParaHit-f kit test Rapid diagnostic tests (RDTs) showing positive result

Fig 2: Results of SD Bioline malaria antigen *Pf/Pv* test for detection of malaria parasite

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Fig 3: Advantage Mal Card antigen detection test kit showing positive results

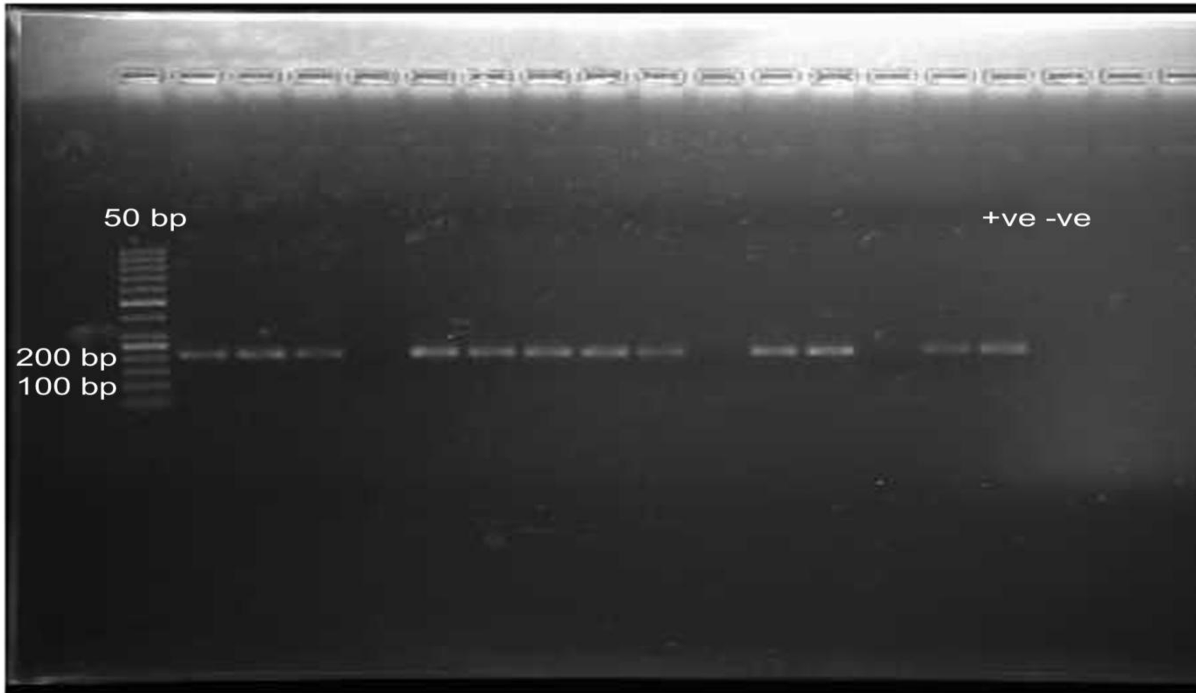

Fig 4: Schematic representation of detection of *Pf* at 205 bp on agarose gel electrophoresis

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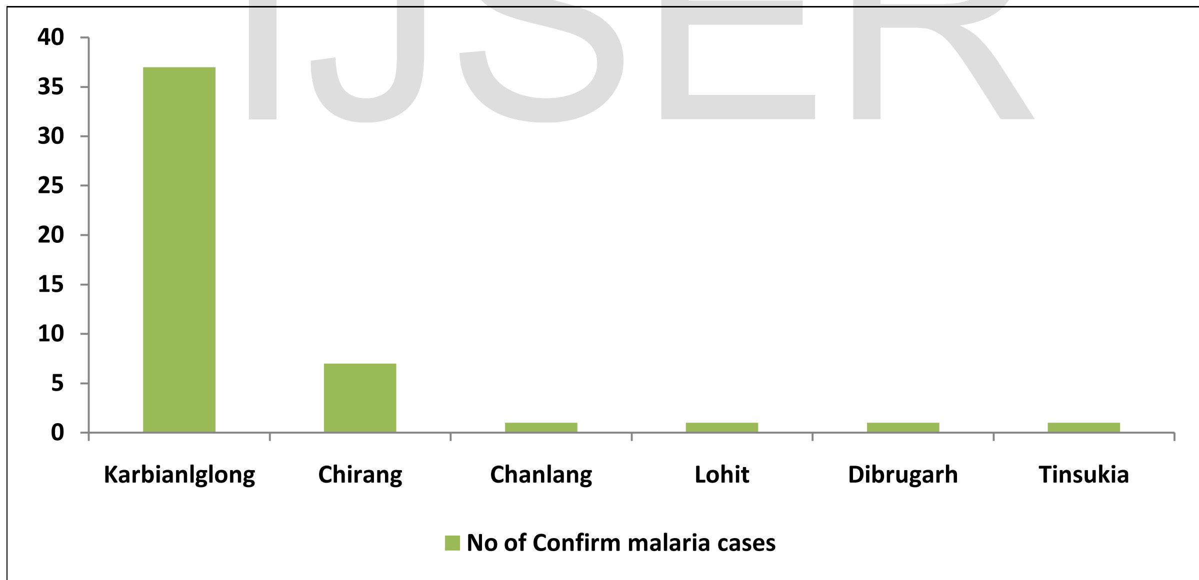

Fig 5: Distribution of malaria cases in different malaria endemic areas of Assam and Arunachal Pradesh

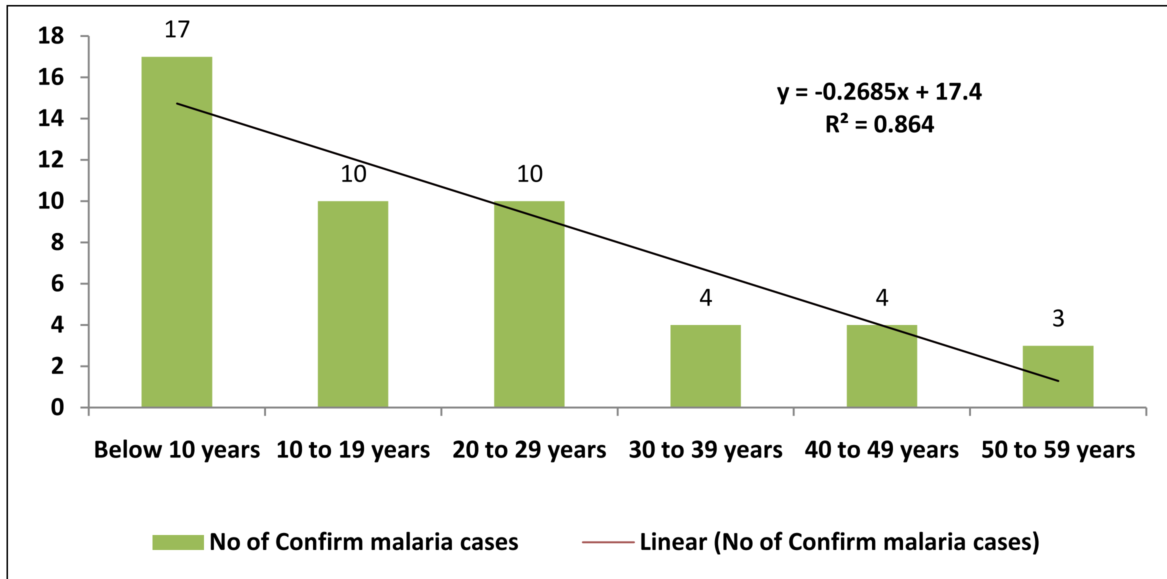

Fig6: Age wise distribution of confirmed malaria cases in different parts of Assam and Arunachal Pradesh

A simple linear regression analysis (y = -.2685x + 17.4) was performed to determine if any significant relationship (P = 0.0072) between increased in age trend with no. of confirmed malaria cases (Fig 7). Conclude that with an increase in the age trend by 1 year, nos. of confirmed malaria cases decreased approximately by 0.26 nos.

4. Discussion:

People of all age groups and sex were found affected with malaria. Malaria was significantly high in the younger age group of <10 yrs. The demographic analysis among the study subjects indicated that there was a decrease in the number of confirmed malaria cases with increasing age. This may be due to the development of immunity against the parasite with increasing age, as those areas are endemic for malaria and the patients having history of it.

Many of the hospitals in rural areas of Assam and Arunachal Pradesh rely on SD Bioline malaria antibody *Pv/Pf* test for detection of malaria and providing antimalarial drugs like Chloroquine to the infected patients. There is every possibility for presence of malaria antibodies within the people as those areas are highly endemic for malaria. Therefore antibody tests will lead to false case interpretation for acute plasmodium infection.

Clinical diagnosis of malaria currently depends on the visualization of parasites by light microscopy of Giemsa-stained thick and thin blood smears. The procedure is cheap and simple, but it is a labour intensive procedure and requires well-trained personnel [10]. In our study, microscopic examination gives 18.75% false negative rate and 4.17% false positive rate. This may be due to diagnostic sensitivities of malarial tests are influenced by the parasite density, environmental conditions etc. Especially in *P. falciparum*, the observed parasitaemia may not correlate directly with the parasite antigen or biomass due to the effect of parasite sequestration[11]. Since *P. falciparum* may be sequestered in the deep capillaries of the spleen, liver, and bone marrow, *P. falciparum* infection may be missed easily in the event of insufficient parasites for detection in the blood films [8, 12]. A previous malarial treatment history or self-medication may also decrease the parasite density [13]. Moreover, the cumulative effect of the antigen concentration could allow antigen detection, while the parasite density remains below the threshold for microscopy [14]. Therefore, an accurate malaria diagnosis could not be achieved.

In contrast to light microscopy, other diagnostic test kit like the *ParaHit-f* test is rapid and technically easy to perform. It takes approximately 10 minutes to perform a single test and we can perform many tests simultaneously.

The value of the ParaHit-*f* sensitivity observed in the present study is very less which shows consistent results with other studies conducted in central India and other parts of the world. [28-31]. The specificity appears to be are not consistent from various areas ranging from 75% to 100% as our study revealed less specificity of it[28-31]. Another study conducted by Kamugisha E et al., the sensitivity and specificity of ParaHit-f were 29.8% and 98.8% respectively which shows that sensitivity is in close proximity to our result but not the specificity , whereas Eliningaya J. et al.,found sensitivity of ParaHit-f to be 10.7% (95% CI, 6.7-14.7) and specificity of 100% (95% CI, 97.4-102) [26.27]. The sensitivity of the other RDTs evaluated in this study is similar to the results of other published studies [32-36]. Surprisingly, all of the RDTs evaluated in this study showed relatively low specificity resulting in high false positive rates which is not acquiescent with most published studies [32-37]. However there are only few studies where they found low specificity which is in accordance with our finding [38, 39].

From the above finding concomitant use of microscopy and other methods is recommended along with rapid diagnostic tests for confirmation of malaria parasitaemia. Similarly SD Bioline malaria Ag P.f/Pan kit test, Advantage Mal Card tests are also reliable to

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used but PCR appears to be a useful method for detecting *Plasmodium* parasites. Similar finding on PCR sensitivity and specificity was found in earlier studies [15-23]. But the other diagnostic method like ParaHit-f kit test shows highest false positive and false negative rate as compared to the other diagnostic rapid tests. False positive tests can occur with RDTs for many reasons. Potential causes for PfHRP2 positivity, other than gametocytemia, include persistent viable asexual-stage parasitaemia below the detection limit of microscopy (possibly due to drug resistance), persistence of antigens due to sequestration and incomplete treatment, delayed clearance of circulating antigen (free or in antigen-antibody complexes) and cross reaction with non-falciparum malaria or rheumatoid factor. Proportion of persistent positivity has been linked to the sensitivity of the test, type of test, degree of parasitaemia and possibly the type of capture antibody etc. On the other hand, false negative tests have been observed which has been attributed to possible genetic heterogeneity of PfHRP2 expression, deletion of HRP-2 gene, presence of blocking antibodies for PfHRP2 antigen or immune-complex formation, prozone phenomenon at high antigenemia or to unknown causes.

Although each method has a different detection threshold; discordant results can result from changes in the parasite densities during the clinical course [8, 24]. However newer, more advanced malaria diagnostics based on fluorescent microscopy and detecting of nucleic acid (PCR) are well known, but there are limitations for these newer require skill, equipments as it is not readily available in many malaria-endemic countries [25]. The result of the present study showed that PCR is more superior followed by Microscopic slide experiment, SD Bioline malaria antigen *Pf/Pan* test, Mal-Card antigen test and, *ParaHit-f* kit test in detection of malaria parasites. Although in the case of positive test results given by microscopic examination and other technique, confirmation by molecular techniques is necessary.

5. Conclusion

The results of the present study showed that SD Bioline malaria Ag Pf/Pan test and Advantage Mal Card test revealed good sensitivity and specificity which was approximately in close proximity to PCR results. Generally different public health centres in Assam and AP were used government supply ParaHit-*f* kit for diagnosis of malaria. We found its less sensitivity and specificity therefore, it is necessary to initiate a new plane for providing accurate diagnostic kit which will help to performed prompt and accurate diagnosis so that a possible recrudescence

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after an incorrect treatment of infected individuals can be avoided. Accurate diagnosis may also reduce the possibility of persistence of malaria load in the endemic areas through which it is possible to overcome from this burden.

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