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Diagnosis of Malaria Infection using Three Diagnostic Techniques in Diary Villages Khartoum North State during the Period October 2015-Febrory 2016

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Abstract

Background: *Laboratory diagnosis of malaria is currently recommended for the confirmation of the disease before management. The two most common techniques in use for the diagnosis of malaria parasite in Sudan are Giemsa stained smears and Rapid diagnostic tests(RDTs). This study was carried out to compare microscopy (Giemsa and Acridine orange stain) and RDTs as effective tools for the diagnosis of malaria among individuals with clinical symptoms attending local primary health care center.*

Methods: *Traditional Giemsa stained thick blood films were compared with Acridine orange fluorescence's techniques and RDTs for the diagnosis of malaria in blood smears collected from symptomatic individual in new malaria area of Diary villages of Khartoum North state, Southern Khartoum Sudan. The information of clinical symptoms were collected by the clinician using structured questionnaire. A total of 432 subjects were examined between the period of October 2015-Febrory 2016.*

Results: *Prevalence rate of malaria infection was not significantly different between sex (P = 0.06). The overall positive cases were found to be 328(75.9%) out of 432. The commonly affected age group was found among children aged 1-10 years old 164 (38%). The study also revealed predominance of Plasmodium falciparum malaria 227(73.9%) among all the positive cases of malaria. Performance of the three techniques Giemsa stain, Acridine orange stain and RDTs showed positive rate 139(42.9%), 266(81.1%) and 306(93.6%) respectively.*

Conclusions: *The study concluded that P. falciparum is the commonest species in the area. The acridine orange diagnostic technique is a valuable alternative method that can be used specially in well equipped laboratories. Rapid diagnostic tests alone can't replace Giemsa stain technique but when used with other techniques can be with better value.*

Keywords*: Malaria parasite. Giemsa, Acridine orange, RDTs.*

Introduction

Malaria is one of the most important parasitic diseases of humans, which caused by plasmodium spp that transmitted through the bite of infected female Anopheles mosquitoes. About 3.3 billion persons are estimated to be at risk of malaria infection of whom 243 million are infected in Africa and nearly 863,000 died of them (WHO 2008). In the Sudan, malaria is a leading cause of morbidity and mortality. The annual estimated number is 7.5 million cases and 35,000 deaths, accounting for 20-40% of the total outpatient attendance and around 40% of admissions (WHO-Sudan 2005). Malaria is prevalent in Khartoum with higher transmission in the peri-urban areas (Elsayed 2000). Early diagnosis and treatment of malaria infections are key factors to reduce malaria-related morbidity and mortality. In many endemic countries including Sudan, patients are usually clinically diagnosed (fever, nausea, joint pain, headache, vomiting) and only a small proportion of malaria cases are tested owing to a lack of diagnostic capabilities, therefore raising a considerable uncertainty surrounding the estimate of the number of cases and deaths.

The definitive diagnosis of malaria in most clinical laboratories depend on the demonstration of malaria parasite in Giemsa stained thick and thin blood films (WHO, 1991). But this method is labor intensive and time consuming for the diagnosis of malaria, in addition it requires a reader with experience and skill to provide an accurate diagnosis (McKenzie FE 2003). Alternative to traditional Giemsa staining of blood film for the detection of malaria parasites Sodeman in 1970 introduced for the first time the staining of

thick blood film by Acridine orange. A few year later WHO proposed the Acridine orange staining for identification of malaria parasite (Shute and Sodeman 1973). Adding of fluorescent dyes to blood film highlighted the presence of low parasitaemia within erythrocytes and it can be considered as a potential method of improving the accuracy of microscopic diagnosis (Srinivasan, 2000). However this method has several limitations including cost and well equipped laboratory. The diagnostic errors in microscopy occurs more commonly with low density parasitaemia (10 – 100 parasite/μl of blood) or in higher density of $>$ 5000 / μl of blood (Kilian, 2000).To overcome this problem new technology methods have emerged and they include antigen detection, serology for antibodies flow cytometry and PCR (Murray,2008). The rapid diagnostic test (RDTS) performance for diagnosis of malaria has been reported as excellent method(Chilton, Donder 2006, 2007). Bell (2006) and Murray (2008) have showed a wide variation in sensitivity in malaria endemic areas. Overall RDTs appear as highly valuable, for healthcare workers for it's simplicity to perform even by unskilled person.

The test employ lateral-flow immunochromatographic technique (Bell 2006). The limitation of RDts is that it can give false positive and false negative results due to the persistence of the target antigens, their inability to distinguish plasmodium species, cost and limited to monitor responses therapy (Sotimehin, 2008). This study was carried out to compare Giemsa-stained smears, Acridine orange stained blood film microscopy and malaria rapid diagnostic test as methods for the detection of *Plasmodium* parasitaemia among patients with fever and other symptom at primary health care centre at the end of rainy season in Diary villages Sudan.

Material and methods

The study area are Diary villages in Northern Rural Province of Khartoum North, located 60 Km North Khartoum and about 3.5 Km from the River Nile. The area cover a land mass of 20 Km², with 3.500 inhabitant of one ethnic group with low socioeconomic status. The inhabitants use piped water which they store in large open container for many weeks. The area is considered as a new malaria area. Ethical approval was obtained from the medical research ethical committee in the National Ribt university. The study was clinical and laboratory based study done in the period of October 2015 - February 2016. A total of 432 patients, both sexes and different age groups with clinical symptoms of malaria who attended the clinic during the study period were included in the study.

Blood collection and examination

2 ml of venous blood samples were collected into an Ethylene diamine tetra acetic acid (EDTA) containing bottles for the study, using vein puncture technique (CEC Okocha, 2005). For malaria microscopy two slides thick and thin smear were made from patient's blood samples according to Cheesebrough (2000), allowed to air-dry and fixed in methanol. One slide smear from each sample was stained with 10% Giemsa solution using standard procedure and examined microscopically under oil immersion at X 100 on Olympus CH25 microscope, by two expert technologist from the parasitology unit faculty of laboratory sciences Ribat university. A film was considered negative if 100 microscopic fields showed no parasites.

For Acridine orange technique Just before examination, 10 μL of Acridine orange stain (100 pg / mL in phosphate - buffered saline, pH 7.2) were pipetted on to a clean 22x22 mm glass cover slip which was then inverted and placed on the prepared blood film. The slide was examined at magnification of x 40, on Olympus microscope with mercury light source model B X 51 TRF Japan, by specialist technologist from the microbiology laboratory. Parasite nuclei fluoresce bright green, while cytoplasm appear yellow orange. Each sample was also subjected to malaria RDTs using the SD BIOLINE Malaria Ag P.f & P.v Test kit (Standard Diagnostics India) according to manufacturer's instructions by trained medical assistance at field. Microscopy and RDTs were performed by different persons and each was blinded from the report of the other. Data obtained was analyzed using the statistical package *SPSS version* 21. The level of significance was set at ≤ 0.05.

Results

Out of the 432 individuals enrolled in the study, 206/432 (47.7%) were males 226/432 (52.3%) were females.

The overall prevalence of malaria among the studied group was 328 out of the 432 patients. The commonly infected age was among the age group 1-10 years (164/328 (38%) table 1.

Table 1 Age prevalence of malaria infection among patients attending the Primary health care

Age range	positive		negative		total
1-10	164	38.0%	57	13.2%	221
11-20	57	13.2%	20	4.6%	77
21-30	47	10.9%	11	2.5%	58
More than 30	60	13.9%	16	3.7%	76
Total	328		104		432

No significant difference was observed in malaria prevalence among the different sexes ($p = 0.06$). Table 2.

Table 2 Sex-related prevalence of malaria infection among patients attending the primary health care

	Frequency	Percent %	Males	Females	P.value
Positive	328	75.9	165 50.3%	163 49.7%	
Negative	104	24.1	41 39.4%	63 60.6%	0.06
Total	432	100	206	226	

Table 3 Comparison of Acridine orange fluorescence examination of thin blood films, Giemsa stained thick blood film techniques and Rapid diagnostic test for diagnosis of malaria

	G stain	%	A O stain	%	RDTs	%
Positive	139	42.4	266	81.1	306	93.6
Negative	189	57.6	62	18.9	22	6.4
Total	328	100	328	100	328	100

Table 4 Comparison of the three techniques and the correlation each other

	positive	Negative	Total	P-value
G.stain	139	171	310	0.000
RDTs	307	125	432	
AO.stain	246	20	266	0.000
RDTs	307	125	432	
AO.stain	266	0	266	0.000
G.stain	139	293	432	

The detection of the malaria parasite infection by the three different techniques showed that the positive results by the rapid diagnostic test was 306/328 (93.6%), by the acridine orange stained thin blood film 266/328 (81.1%), and by the Giemsa stained films as the gold standard 139/328 (42.9%) (table 3). Acridine orange stain and RDTs showed sensitivity and specificity of 100%, 98.9% and 71.0%, 61.7% respectively. All the positive specimens by AO stain were positive also by the Giemsa stain. There were twenty positive specimens by A O stain that were negative by RDTs and three specimens that were positive by G T but negative by RDTs.

The correlation between the three techniques using the Giemsa stained smears as the gold standard showed a significant difference in favor of the rapid diagnostic test and the acridine orange stain (P = 0.000) (table 4).

The distribution of the different plasmodium species diagnosed by the rapid diagnostic test among the 307 positive results was *plasmodium falciparum* 227, *plasmodium vivax* 43 and mixed infection 39.

Discussion

All the study samples were collected from patients with clinical symptoms of malaria in Diary villages. The study compared three techniques to detect malaria parasites, and it revealed a high malaria prevalence rate (75.9%) among patient attending the primary health care center in the village, which is high compared to the study done in Dibaira camp in New Halfa town Sudan (Yousif *et al*., 2005). Another study done in Dar Alsalam and Jabal Awlia camp showed also low prevalence 5% and 11% respectively (Miskelyemen A *et al* .,2012). The explanation for this high finding may be due to the changing of the population habits. In the past they use to bring water from the River Nile which is far from the village. Now a days they use pipe water and they usually store it in large open containers together with small pools that created by broken pipes as well as the activity of cultivation of some vegetables around the habitat which created a good condition to the vector breeding.

Compared to this report Houmsou reported high prevalence rate of (39.5%) of malaria among patients in Nigeria and Chansuda and Awalludin 48% in the Republic of Indonesia (Houmsou *et al*., 2011, Chansuda and Awallndin (2006). The highest malaria prevalence was observed in patients of the age groups 1-10 year with 38.0%. This probably due to the weak immunity status of this group and their ignorance of the preventive measurement. This finding is lower than that reported by Alioune *et al*. who observed 51.8% among the age groups 1-13 years and 55-68 years in Dielmo and Ndiop villages of Senegal (Alioune *et al*., 2010). The similar rates of infection observed among males (50.3%) and females (49.7%) could be the result of exposure to malaria parasite due to environmental and living conditions which support the availability of the vector.

Performance of the HRP-2 Rapid Diagnostic Test recorded a sensitivity of 98.9% and specificity of

61.7.4%. Sensitivity and specificity obtained in this study were within the range that found by previous studies ((Houmsou *et al*., 2011, Willcox ML *et al*., 2009, Murray CK *et al*.,2008) The false-negative result obtained (6.1%) could be due to undetected HRPT-2 antigen which may be due to gene deletion by individual for the production of HRP-2 and so will give a negative result with these RDTs (WHO, 2008). Other limitations of RDTs for this antigen is related to the method of storage and transport which affect the test sensitivity (WHO, 2008).Other limitation of RDTs is the false negative results. In this study there were 3 specimens gave negative RDTs but the parasites were detected in Giemsa stained films. Also 20 specimens were negative in RDT but positive in acridine orange fluorescence techniques. Films examination in microscopy remains the standard method for diagnosing malaria. However in settings where microscopy is unavailable the application of RDTs can be of very high value in the diagnosis of malaria specially if other RDTs that detect other target antigen such as PLDH or Aldolase are used.

In case of Acridine orange staining the study result demonstrated the highest sensitivity 100% and specificity 71.0% compared to the Giemsa staining. This was different from other study that reported sensitivity and specificity 96.4% % and 95.1% respectively (F&d&rick Gay., *et al* 1996). One hundred and twenty seven specimens positive by AO were negative by G T. So the advantage of Acridine orange stain is that it is more sensitive than Giemsa stained smears. However, the technique necessitates a fluorescence system, which is not usually available in most of the developing counties. To overcome this problem daylight-illuminated microscopes fitted with interference filters, could have significant advantages for field use Kawamoto, F. (1991).

The current study documented that *P. falciparum* is the most common species diagnosed from the cases reported in the primary health care center, followed by *Plasmodium vivax.* No other species detected among the inhabitant of the Diary villages. This high occurrence of *P. falciparum* was reported by other investigators in Khartoum state and the findings of WHO which reported predominance of *P. falciparum* infection in sub-Saharan Africa ((Miskelyemen A *et al*., 2012, WHO, 2008)

Conclusion

The results concluded that malaria infection was common among symptomatic individuals attending the primary health care , and *P. falciparum* was the common infective species. Although the RDTs has some limitations due to the false negative results but it can be a good diagnostic method in settings where microscopy is unavailable. However blood film examination by microscopy remains the standard method for diagnosing malaria since it is sensitive , cheap and simple to apply. The study showed that A O was quite sensitive even in specimen with low density of parasites, Combination of A O and RDT could be an appropriate method for both clinical and epidemiological studies. The study recommended that A O using modified light microscopy should be applied in parallel to G T or/ and RDT.

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