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ESTIMATION OF PLASMODIUM FALCIPARUM AMONG HIV PATIENTS IN KHARTOUM –SUDAN

Parasitology

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ABSTRACT

Malaria and HIV are among the two most important global health problems in developing countries. They cause more than 4 million deaths a year. Since 2009, the CDC has included malaria in the list of AIDS-related opportunistic infections; even though malaria is not among the leading causes of death in HIV-infected patients, it has been identified as the third most important source of HIV-related morbidity in Africa. The study was a cross-sectional study to detect *P.falciparum* among HIV patients and was conducted at the Omdurman center for volunteer council test and AL Khartoum center, Khartoum state, Sudan public from December 2017 to March 2018. A volume of 3- 5 ml blood sample was obtained by venipuncture from each of these patients into an ethylene diamine tetra-acetic acid (EDTA) anticoagulant bottle and centrifuged to obtained serum for nested PCR examination.DNA extracted by Patho Gene-spin™ DNA/RNA Extraction Kit and P.falciparum was detected by nested PCR. A total of 70 patients were randomly selected in the study with the (38%) were females. Most of the patients were on stage III (76%). The majority (98.6%) of the patients was negative for the *Plasmodium falciparum* and only one case (1.4) was positive for the *Plasmodium falciparum*. Our result showed the positivity occur in stageIII and the relationship between the *P.Falciparum* and gender was significant as the P value =0.46≤ (P=0.05).

KEYWORDS

INTRODUCTION:-

Malaria and HIV are among the two most important global health problems in developing countries. They cause more than 4 million deaths a year [1]. Since 2009, the CDC has included malaria in the list of AIDS-related opportunistic infections; even though malaria is not among the leading causes of death in HIV-infected patients, it has been identified as the third most important source of HIV-related morbidity in Africa [2], In fact, HIV infection is expected to increase the morbidity and mortality attributed to malaria, since immune suppression impairs the immune response to Plasmodium, determining more frequent occurrences of clinically severe malaria [3]. Both malaria and HIV can cause hematological abnormalities independently. Those hematological abnormalities: anemia, thrombocytopenia, and leucopenia have been documented as strong, independent predictors of morbidity and mortality in malaria co-infected HIV positive individuals than monoinfected HIV positive individuals. Beside this HIV infection is associated with a twofold higher risk of severe malaria in adults, and a six to eightfold increase in the risk of death [4]. There is growing evidence that the two infections may synergistically intensify each other, increasing incidence and complicating treatment efforts. In a region of unstable malaria, HIV infection had an unexpectedly large association with the outcome of falciparum malaria where HIV infection was associate with severe/complicated malaria [5].

Malaria is caused by the protozoan parasite Plasmodium and is transmitted by Anopheles mosquitoes. It is endemic in most tropical and subtropical regions of the world [6] and remains one of the leading causes of morbidity and mortality globally and nearly half of the global populations are at risk of malaria infection [7]. Malaria in humans is caused by 5 Plasmodium parasites: *Plasmodium falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi*. The current distribution of human-pathogenic Plasmodium species shows a preponderance of *P. falciparum* in tropical Africa, while *P. vivax* prevails over P. *falciparum* in South America. Both *P. falciparum* and *P. vivax* are prevalent in south-eastern Asia and western Pacific [8]. malaria infection is associated with strong CD4+ cell activation and up-regulation of proinflammatory cytokines, it provides an ideal microenvironment for the spread of the virus among the CD4 + cells and thus for rapid HIV-1 replication[1].HIV infection is a global pandemic. By the end of 2007, it was estimated that about 33.2 million people were living with HIV in the world with more than 60% of the infected population in sub-Saharan Africa [9]. Human Immunodeficiency Virus (HIV) is a retrovirus that can infect immune competent causing an impairment of host defense [10]. It is also a potential means by which HIV affects disease course and outcome in other infections, such as malaria [6]. HIV replication triggers the continuous destruction of CD4+ T helper and this is compensated by steady replenishment of the T-cell pool, which leads to the break-down of the immune system and the concomitant development of immunodeficiency [11]. HIV has a critical role in both Th1-type and Th2-type responses to malaria and T-cell activation in co-infected patients can worsen the immune response to both diseases [12]. Malaria is conventionally diagnosed by microscopic examination of stained blood films using Giemsa, Wright's, or Field's stains [13], QBC technique: This method involves staining parasite deoxyribonucleic acid (DNA) in micro-hematocrit tubes with fluorescent dyes, e.g. acridine orange[14], Rapid diagnostic tests (RDTs): RDTs appears a highly valuable, rapid malaria-diagnostic tool for healthcare workers, In malaria-endemic areas where no light microscopy facility exists that may benefit from RDTs[15], Serological tests: usually based on the detection of antibodies against asexual blood stage malaria parasites. Immunofluorescence antibody testing (IFA) has been a reliable serologic test for malaria in recent decades[16], PCR technique:PCR can detect as few as 1-5 parasites/µl of blood (≤ 0.0001% of infected red blood cells) compared with around 50-100 parasites/µl of blood by microscopy or RDT[17,18], Microarrays: The principle of the microarrays technique is Hybridization of labeled targets divided from nucleic acids in the test sample to probes on the array enables the probing of multiple gene targets in a single experiment[19], FCM assay: is based on detection of hemozoin, which is produced when the intra-erythrocytic malaria parasites digest host hemoglobin and crystallize the released toxic heme into hemozoin in the acidic food vacuole[20,21], Mass spectrophotometry: cleanup of whole blood samples, followed by direct ultraviolet laser desorption mass spectrometry (LDMS)[22], LAMP technique: a simple and inexpensive molecular malaria-diagnostic test that detects the conserved 18S ribosome RNA gene of P. falciparum[23].

METHODS

The study was cross -sectional study to detect *P.falciparum* among HIV patients and was conducted at the Omdurman center for volunteer

council test and AL Khartoum center, Khartoum state, Sudan public from December 2017 to March 2018. The study population was 70 patients confirmed to be HIV-seropositive by standard laboratory techniques. The study protocol was submitted for the agreement of the Omdurman center for volunteer council test and AL Khartoum center administration. Informed consent was obtained from all participants - after explaining the aim of the study to the participant prior to their enrolment in the study. A volume of 3- 5 ml blood sample was obtained by venipuncture from each of these patients into an ethylene diamine tetra-acetic acid (EDTA) anticoagulant bottle and centrifuged to obtained serum for nested PCR examination.DNA extracted by Patho Gene-spin™ DNA/RNA Extraction Kit and *P.falciparum* was detected by nested PCR.

DNA extraction:
DNA extraction was done at room temperature in the fume hood. All materials like tubes pipette –tips, and Pasteur pipettes were sterilized before use. The blood was transferred to a 15 ml falcon tube and was well mixed by inversion with 10 ml of red cell lysis buffer. The samples were then spied for 10 min at 3000 rpm in a clinical centrifuge to pellet the white blood cells. The supernatant was discarded and the cell pellet was washed aging with RBCL till pellet was completely white then 1.8 of WBCL buffer + 10µl proteinase K + 4µl guanidine were added to the pellet , vortexed and mixed thoroughly and left to digest overnight at 37C. The digested mixture was transferred to Eppendorf tube .An equal volume of chloroform was added then shook 10 times and centrifuged at 1400 rpm for 3 min. The aqueous phase (upper layer) that contained the DNA was transferred to a new Eppendorf tube and 2 volume of ice-cold absolute ethanol was added. The tube was left overnight at-20C◌̊. The DNA was spun down at 14000 rpm for 20 min. The supernatant was discarded and then the pellet was washed with 70% ethanol at 14000 rpm for 20 min. The supernatant was discarded. The pellet was left to dry for 30 min at room temperature and then was suspended in 100 µl dd water and left at 4 C◌̊ for 24 hrs to dissolve

PCR analysis:
Nested PCR amplification of the small subunit ribosomal RNA gene, was done to detect the parasite DNA, following the methods described by Snounou et al., (1993). [24]. PCR reaction was carried in a total of 20 ul reaction. Amplification was performed in 50mMKCl/10mM Tris pH8.3, 125 uM of each of the deoxyribonucleotide triphosphate, 1.0 unit of Taq polymerase (Perkin Elmer Cetus) and 1 ul of the template DNA was used per reaction.The gene was amplified by nested PCR, each amplification with conserved or family – specific primer pair done separately. The steps of the amplification program were as follows: step 1: 95 Ċ for 5 min, step 2: 55 Ċ for 2 min, step 3: 72Ċ for 2 min, step 2-4 were repeted 39 times, then step 2 and finally step 3 for Control positive and negative P.falciparum blood samples were included on the PCR analysis.

Agarose gel electrophoresis:
2% of the gel was prepared in a conical flask by adding 1g of agarose to a 50 ml 1X TBE and was then swirled to mix. The agarose was heated to 100ċ and was then left to cool on the bench until the temperature reaches 60Ċ. 3µl of ethidium bromide was added and swirled to mix. The gel was slowly poured into the tank and then was left to sit for 30 minutes. 250 ml of 0 .5 x TBE were added into the gel tank 5µl of the sample were transferred to the parafilm and then 5µl of loading buffer was added. The gel tank was closed and the power –switch was set on

PCR reaction mix:

Component	Volume
H_2O	12µl
5x FIREPol Master MIX Ready o Load	4µl
Forward Primer(10 Pmol/µl)	1µl
Reverse Primer (10 Pmol/µl)	1µl
DNA template	2µl
Total reaction Volume = 20µl	

Two amplification reaction Nest1 & Nest 2

- In the first amplification reaction (Nest 1) used a pair of oligonucleotide primers (rPLU5 and rPLU6) to detection genes of any plasmodium parasite, the product of this reaction used as DNA template for second amplification reaction (Nest 2).
- In the second amplification reaction (Nest 2) used Oligonucleotide primers specific to Plasmodium species: rFAL1 and rFAL2 for P.falciparum (product of **206 bp**).

Table: The Amplification Reaction Nest 1 (table 1):

Steps	Temp	Time		Cycle
Step1	94ºC	0:02:00		
Step2	94 ºC	0:00:30		
Step3	55 ºC	0:01:00		
Step4	72 ºC	0:01:00	2	40
Step5	58 ºC	0:02:00		
Step6	72 ºC	0:05:00		

Table 2: The Amplification Reaction Nest 2 (table 2):

Steps	Temp	Time		Cycle
Step1	94 ºC	0:02:00		
Step2	94 ºC	0:00:30		
Step3	58 ºC	0:00:40		
Step4	72 ºC	0:01:00	2	40
Step5	58 ºC	0:02:00		
Step6	72 ºC	0:05:00		

- **Agarose Gel preparation 2%**

Statistical analysis:
Statistical analysis of the data was performed using SPSS. Variables descriptively expressed as number and percent.

RESULT

Characteristics of the study group
A total of 70 patients were randomly selected in the study with the (38%) were females. Most of the patients were on stage III (76%).Table (3)

Table 3: Characteristics of the study group

Variables	Frequency	Percentage (%)
Gender		
Male	32	45.7
Female	38	54.3
Age		
3-18 Years	4	6.0
19-36 Years	32	46.0
>36 Years	34	48.0
Stage of disease		
Stage I	14	20.0
Stage II	3	4.0
Stage III	53	76.0
Total	**70**	**100.0**

The majority (98.6%) of the patients was negative for the *Plasmodium falciparum* and only one case (1.4) was positive for the *Plasmodium falciparum*. Table (4)

Table 4: frequency and percentage of *P.Falciparum* among Patients

Variable	Frequency	Percentage (%)
Positive	1	1.4
Negative	69	98.6
Total	**70**	**100.0**

Our result showed the positivity occur in stageIII and the relationship between the *P.Falciparum* and gender was significant as the P value =0.46≤ (P=0.05) (table 5).

Table 5: The rate of *Plasmodium falciparum* regarding to stage of disease, age and gender

Stage of disease	P.Falciparum		Total
	Positive	Negative	
Stage I	0 (0.0%)	14 (100.0%)	14
Stage II	0 (0.0%)	3 (100.0%)	3
Stage III	1 (1.9%)	52 (98.1%)	53
Total	1 (1.4%)	69 (98.6%)	70
P-value	0.850		
Age	P.Falciparum		Total
	Positive	Negative	
3-18 Years	0 (0.0%)	4 (100.0%)	4
19-36 Years	0 (0.0%)	32 (100.0%)	32
>36 Years	1 (2.9%)	33 (97.1%)	34
Total	1 (1.4%)	69 (98.6%)	70
P-value	0.584		

Sex	P.Falciparum		Total
	Positive	Negative	
Male	1 (3.1%)	31 (96.9%)	32
Female	0 (0.0%)	38 (100.0%)	38
Total	1 (1.4%)	69 (98.6%)	70
P-value	0.457		

DISCUSSION

HIV-infected patients are at higher risk of parasitic (opportunistic and non-opportunistic) infections including malaria because of their suppressed immune systems. Malaria and HIV are among the two most important global health problems in developing countries. They cause more than 4 million deaths a year. In the current study, only one case (1.4%) was positive for the *Plasmodium falciparum* as compared with Onyenekwe et.al (2007) (24) and Tay et.al (2015) (6) those showed 45.15% and (11.75%) respectively. The previously mentioned studies which are tabard with this study due to the difference in transmission rate during the time of collection and sample size In this study there was statistically significant difference between the malaria prevalence rate of females (0.0%) and males (3.1%) P = 0.457 this disagree with Tay et.al (2015) (24) as There was no statistically significant difference between the malaria prevalence rate of females (12.1%) and males (10.2%) P = 0.6047.

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