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Relevance of the Widal Agglutination Test in Malaria Endemic Region

Onemu, S.O.,[1*] Egbe, E.C.[2] and Omoregie, R.[3]

[1] Faculty of Medical Laboratory Science, College of Basic Health Sciences, Achievers University, Owo. Nigeria.

[2,3.]Department of Microbiology University of Benin Teaching Hospital

Corresponding author e-mail: onemuso@achievers.edu.ng; samsononemu@gmail.com

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Abstract

Malaria and typhoid fevers are twin diseases that plague many Sub-Saharan African and Asian countries at epidemic proportions with almost a third of all deaths occurring in Nigeria in the last decade. Diagnosis of malaria in Nigeria relies heavily on the simple and inexpensive microscopy and the Widal agglutination test is commonly used in resource-poor settings where cultures fail or unavailable for typhoid fever diagnosis. The study was conceived to determine the relevance of the Widal agglutination test in a malaria endemic region. Patients who had previously received artemisinin combination therapy (ACT) with unremitting febrile illness who gave consent were enlisted into the study. Blood samples were collected for blood films, examined microscopically for the presence of malaria parasites. Widal agglutination test was carried out on each separated serum with the rapid slide agglutination test and titre was determined for sera showing agglutination with tube dilution assay. Malaria parasites were present in 236/429(55.0%) of the blood samples of which 41.7% had parasitemia density of ≤5000/mL of blood. Parasitemia density higher than 5000/µL of blood accounted for 13.4%. Widal agglutination titres as high as 320 were recorded in patients with malaria parasites as wells as those without malaria parasitemia. Malaria is a major cause of febrile illness in the study population which can be carefully excluded with the inexpensive blood film microcopy to enable proper evaluation for typhoid fever to be undertaken.

Keywords: Agglutinins, malaria endemicity, typhod fever, Widal agglutination test.

1.0 Introduction

Malaria and typhoid fever are two major diseases that are still prevalent in most topical countries of Sub-Sahara African and Asian nations. Several millions of people residing in these endemic areas contract these diseases (Verma *et al*, 2004). Typhoid fever has been recognized as a leading cause of many health problems in developing countries including Nigeria that results in morbidity (Mano *et al*., 2007; Andvalem *et al.*, 2014). The clinical symptoms of both malaria and typhoid fevers is usually manifested as febrile illness or pyrexia of unknown origin (P.U.O). Typhoid fever is an acute infectious disease caused by different *Salmonella* species but classically by *Salmonella typhi*, *S. typhi* is responsible for epidemic outbreaks particularly in the Indian sub-continent and South-East Asia (Krapizak *et al.*, 2002).

The gold standard established for the diagnosis of typhoid fever is dependent on the isolation of Salmonellae from clinical samples (Zongani and Ziglam, 2004) or the detection of rising or falling titres of agglutinins (antibodies) in serum (House

et al., 2001; Adesegun *et al*., 2020). The Widal agglutination test could be used for the diagnosis of typhoid fever in patients who have clinical indications of typhoid fever but cultures turn out to be negative or in resource poor settings where culture facilities are poor or unavailable (Taiwo *et al.*, 2007).

The morbidity and mortality associated with malaria infection makes the disease a major public health issue (Autine *et al*., 2012; Slater *et al*., 2022). The contribution of Sub-Saharan African countries to the global burden of malarial infection stands at 95% in the last one decade (Oboh *et al*., 2022). Nigeria alone accounted for over a quarter of the 32% malaria related deaths globally in the year 2020 (WHO, 2021). Malaria is a top ranking killer of children under the age of 5 years in Nigeria (Dasgupta *et al*., 2022) as well as responsible for the highest population wide number of malaria cases globally (Dawaki *et al*., 2016). Malaria is a blood-borne parasitic protozoan infection transmitted typically by mosquitoes (Marsh, 1998). Five main *Plasmodium* species regularly infect man, however, in South-East Asia, a simian species or *Plasmodium knowlesi* has emerged as an equally important cause of human malarial disease

2.0 Materials and Methods

Consecutive patients with requests for Widal agglutination test for which artemisinin based combination therapy (ACT) had been administered at different medical centres in Benin City with persisting complaints of febrile illness who gave informed consent were included in the study. The study population consisted of 429 subjects (231 males and 198 females) with age range of 6 months of 72 years.

2.1 Specimen collection

Venous blood (4.0 mL) was collected from each subjects, 2.0 mL was dispensed into ethylene diamine tetra-acetic hydrochloride (EDTA) bottle, mixed by gentle inversion. The remaining 2.0 mL of blood was dispensed into a plain bottle and allowed to clot and the resulting serum separated into another labeled plain bottle.

(Barber *et al.*, 2017). *Plasmodium falciparum* is the most virulent species that is reported for majority of the burden of disease and fatality associated with malaria infection (Zekar and Sharman, 2021), and also the most common cause of malaria in Africa (Nkumana *et al*., 2012).

The emergence of resistance to the commonly used antimalarial agents prompted the introduction of derivatives of artemisinins combination therapy (ACT) to improve and guard against the development of resistant strains of malaria parasites. The first reports on resistance to ACT occurred in South-East Asia (O'Brien *et al*., 2011; Wongstrichanalai and Sibley, 2013). Similar observations have since been made in Africa (Balikagala *et al*., 2021; Manirakiza *et al*., 2022: Tumwenbaze et al., 2022) The gold standard for the diagnosis malaria is the simple and inexpensive blood film microscopy in comparison to the use quantitative buffy coat, QBC or polymerase chain reaction, PCR which are more capital intensive (Slater *et al*., 2012). The study was carried out to determine the relevance of the Widal agglutination test in a malaria endemic region.

2.2 Examination for malaria parasites

Each venous blood was well mixed and 10 μL of blood was taken onto a clean microscope slide and spread with an applicator to give a 1 cm x 2 cm blood film and allowed to dry at room temperature. The dried blood smear was stained with 1% Giemsa stain for 45 minutes.

The film was then gently washed in coupling jar of fresh tap water and allowed to drain dry at room temperature at an angle of 45^{o} on a rack. Each stained blood film was subsequently examined with x100 microscope objective lens for the presence or absence of malaria parasites in at least 100 fields of microscope view. White blood cells count was carried out with a Hematology analyzer (Beckman-Dickinson^{R}). The total number of malaria parasites when present relative the number of white blood cells were carefully noted for the calculation of the parasitemia density (Cheesebrough, 1992). A

thick blood film was made from all positive slides, dried in air an4 stained with Leishman stain for differentiation of malaria species.

2.3 Widal agglutination tests

A rapid slide agglutination test was carried out using the kit manufacturer's instruction (Chromatest, Spain). With each serum using concentrated salmonella antigen of groups A, B, C, and D, 40 μL of serum was taken to equal amount of antigen. This was rock-mixed for 3 minutes. All samples with agglutination reaction were diluted in physiological saline (0.85% sodium chloride) 1 in 20, 40, 80, 160, 320 …. 1280 in 1.0 mL volumes in 75 x 12 mm glass tubes. To each tube was added 40 uL of well mixed concentrated antigen indicated in the slide reaction. The positive control sample included in the kit was treated in a similar manner to validate the test. These preparation were incubated in a water bath set at 37^{o}C for 24 h. The highest dilution of serum showing agglutination was recorded at the titre of the serum.

3.0 Results

The examination of 429 blood samples from subjects with febrile illness showed that 236(55.1%) of the patients had persisting malaria parasitemia. The malaria parasites density in blood of patients is presented in Table 3.1. The highest proportion of patients with malaria parasitemia (41.7%) occurred in patients with malaria parasitemia density of ≤5,000 μL of blood. Parasitemia density of 500l-10,000/mL was recorded in 8.9% of samples while the parasitemia density in the range of 10,001-15,000 was recorded in 3.5% of the samples. Malaria parasitemia density greater than 15,000/uL accounted for only 1.0%. There was no significant difference (X^2, $p > 0.05$) in the degree of parasitemia between males and females.

The age-distribution of malaria parasites is shown in Table 3.2. Malaria parasitemia was highest in patients who were younger than 6 years or 6 years old (47.8%) This frequency was followed by patients in the age-range 7 to 18 years (28.4%). There was a steep decline in parasitemia with increase in the age of patients, especially those who were 40 years or older.

Table 3.3 represents the comparison of Salmonella agglutinins in the sera of patients. Flagella (H) agglutinins at a titre of 160 ranged in proportion from 2.1% to 6.4% for D-H in patients with malaria parasitemia and at a titre of 320, the proportions were 4.1% to 20.7% for D-H agglutinins in patients without malaria parasitemia. The somatic (O) agglutinins at a titre of 160 in these patients were 33.5% and 53.9% and 14.8 to 33.2% at a titre of 320 respectively in patients with malaria and in those without malaria parasitemia. A higher number of patients with febrile illness without malaria parasitemia had agglutinins to group D-O antigens at titres of 160 and 320 than in patients with malaria parasitemia. *Plasmodium falciparum* was the only malaria species identified during species differentiation.

Table 3.1. Malaria parasitemia density in blood of patients (/μL)

Parasitema /u L	No. of Cases	Males	Females
≤ 5000	179(41.7%)	98(22.8%)	81(18.9%)
5001-100,000	38(8.9%)	21(4.9%)	17(4.0%)
10,0011-15000	15 (3.5%)	9(2.1%)	6 (1.4%)
15, 000	4(1.0%)	2(0.5%)	2(0.5%)

Table 3.2. Age distribution of malaria parasites in the study population

Age Range (years)	No. of Cases (%)
≤ 6.0	113(47.8)
7-18	67(28.4)
19-39	44(18.6)
40-59	10(4.2)
≥60	2(1.0)

4.0 Discussion

Malaria parasitemia was present in 236/429 (55.0%) of all the febrile patients studied which may infer that malaria remains a major cause of febrile illness in these patients since they had been previously treated with ACT antimalarial agents. The failure of treatment could have resulted from factors ranging from failure of patients to comply with the prescribed dosage regimen, sub-standard ACT antimalarial drugs which is not uncommon in Nigeria and, or more importantly, as evidence of malaria parasites resistance to ACT that has been reported in Cambodia and Thailand (O'Brien *et al*., 2011; Wongstrichanalai and Sibley, 2013) and more recently in Africa, especially in Uganda (Balikagala *et al*., 2021; Manirakiza *et al*., 2022: Tumwenbaze et al., 2022).

Table 3.2. Comparison of Salmonellae agglutinins in Sera of Patients

Category of Subject	Agglutinin titre to salmonellae group antigens							
	160				320			
	A-H	B-H	C-H	D-H	A-H	B-H	C-H	D-H
Febrile illness with malaria parasitemia (n=236)	5(2.1%)	5(2.1%)	5(2.1%)	15(6.4%)	5(2.1%)	10(4.2%	2(1.0%)	7(3.0%)
Febrile illness without malaria parasitemia (n=193)	8(4.1%)	24(12.4%)	0(0.0%)	40(20.7%)	0(0.0%)	8(4.1%)	0(0.0%)	8(4.1%)
	A-O	B-O	C-O	D-O	A-O	B-O	C-O	D-O
Febrile illness with malaria parasitemia (n=236)	2(1.0%)	17(7.2%)	(5(2.1%)	79(33.5%)	0(0.0%)	5(2.1%)	0(0.0%)	35(14.8%)
Febrile illness without malaria parasitemia (n=193)	0(0.0%)	24(12.4%)	16(8.3%)	104(53.9%)	0(0.0%)	0(0.0%)	0(0.0%)	64(33.2%)

Malaria parasitemia in patients 6 years old or younger accounted for almost half (47.8%) of the population of patients with persisting malaria parasitemia which may infer that the risk of fatality is highest in this age bracket. This finding simulates reports from earlier studies done in

Nigeria (Dasgupta *et al*., 2022). The parasitemia density was generally low with 41.7% of patients having a malaria parasitemia density of ≤5,000/μL of blood. This is also an equivalent of less than one malaria parasite per ten high power fields (HPF, x1000) in thick blood films with the light microscope. This may have been influenced by the prior use of antimalarial drugs that may have eliminated the susceptible phenotypes of malaria parasites, thereby selecting the resistant mutants that persist and continue the infection process.

Subjects with parasitemia in the range of 5001-10,000/μL of blood represented only 8.9% of the population and only 1.0% showed parasitemia density in the range of 10,001-15,000/μL of blood. This may suggest that heavy parasitemia in this category of patients are most unlikely. The degree of malaria parasitemia between males and females was not significantly different ($p>0.05$). *Plasmodium falciparum* was the only species identified during this study infers that, this species is the dominant cause of malaria fever in this population. This is in concordance with prior observations that *Plasmodium falciparum* is the most virulent and commonest cause of malaria fever in Africa (Nkumana *et al*., 2012: Zekar and Sharman, 2021).

The detection of Salmonellae agglutinins in the sera of subjects with malaria parasitemia at titres of 160 is a direct pointer to the possible difficulties in identifying which patients have typhoid fever as such level of agglutinins have also been reported from apparently healthy non-febrile individuals (Ornuse *et al.,* 2010; House *et.al*., 2011; Ganjai *et al*., 2013; Tula *et al.,* 2018). This therefore, diminishes the value and reliance of the Widal agglutination test when malaria parasitemia has not been excluded. This further exacerbates the dilemma in resource poor settings where isolation of the *Salmonella* species is almost impracticable. Agglutinins to H-antigens to salmonella groups A, B, and C were commoner in patients with malaria parasitemia than in those without malarial infection. This may show that agglutinins to H-antigen with titres as high as 160 may be recorded in subjects without typhoid and paratyphoid fevers. However, the higher percent of titre of agglutinins against Salmonella group D-O antigens where *Salmonella typhi* which is a major member of the group and the most frequent organism traceable to most cases of typhoid fever in comparison to other groups of salmonellae.. This may further infer that *S. typhi* is commoner in the study population than Salmonellae in groups A, B or C. However, it is important to take a note of caution that a negative Widal agglutination test also has a level of value in excluding typhoid fever (Andvalen, 2014).

5.0 Conclusion

A high level of malaria parasitemia in febrile patients in the study population was also accompanied with high titres of Salmonellae agglutinins, makes it difficult to clearly differentiate malaria infection from typhoid fever especially with the evidence of emerging malaria parasites resistance to ACT. Exclusion of malaria infection is an important step for the proper diagnosis of typhoid fever in this population and to have a better understanding of the new challenges posed by resistant malaria parasites towards the development of more effective control and elimination strategies.

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