Alneelain University

Graduate College

Colorimetric Detection of Gestational vivax Malaria in Urine Using MSP10 Oligonucleotides and Gold Nanoparticles in Khartoum State

By

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Supervisor

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Background

Malaria is a life-threatening disease caused by parasites that are transmitted to people through the bites of infected female Anopheles mosquitoes which bite mainly between dusk and dawn [1]. It is endemic across Asia, the South Pacific, North Africa, Middle East, and South and Central America [2]. Currently, an estimated 2.9 billion people live at risk of it infection [3]. Research in malaria has been primarily focused on P.falciparum, the fatal form of malaria. However, P.vivax can also cause severe illness with serious complications and costs, especially in children, in whom it has a major impact on growth [4-6].

Women inhabiting malaria endemic areas are at variable risk of acquiring gestational malaria according to the degree of endemicity and their parity. In regions of high stable transmission, they might acquire significant clinical immunity before pregnancy, and placental malaria is often asymptomatic, but it can produce severe maternal anaemia and foetal growth restriction [7]. Approximately, half of the world's population is at risk of malaria but most malaria cases and deaths occur in sub-Saharan Africa. Despite malaria infection can affect all ages and sexes, morbidity and mortality related to malaria are very common in pregnant women and children less than five years of age [8, 9]. It has been estimated that malaria during pregnancy is responsible for 5–12% of all low birth weight and 35% of preventable low birth weight [10] and contributes to 75 000 to 200 000 infant deaths each year [11].
Pregnant women are more susceptible than not pregnant women to malaria, and this susceptibility is greater in first and second pregnancy. Susceptibility to pregnancy-associated malaria probably represents a combination of immunological [12] and hormonal changes associated with pregnancy (Although the nature of the latter is the subject of debate [13]), combined with the unique ability of a subset of infected erythrocytes to sequester in the placenta. Extensive evidence confirms that antibodies directed against the surface of infected erythrocytes in the placenta are important in protection, and are usually absent in first pregnancy.
In malaria endemic areas, at least one in four pregnant women has evidence of peripheral or placental malaria at delivery [14]. It has been documented that malaria parasites may be detected in the placenta of women who are otherwise apparently uninfected; with a peripheral parasitaemia undetectable by microscopy [15, 16].
Malaria and pregnancy are mutually aggravating conditions. The physiological and the pathological changes due to malaria have a synergistic effect on the patient which causes life difficult for the mother, the fetus and the treating physician.
Pregnant women compared to non pregnant women are at an increased risk of malaria and the severity of clinical manifestation experienced by these women and their fetuses depend on the level of pre-pregnancy immunity [17]. Placental parasitaemia in pregnant women leads to impairment of foetal nutrition and this contributes to low birth weight, a leading cause of poor infant development and survival [18]. All the placental tissues exhibit malarial pigments, which obstruct oxygen and nutrient transfer and can cause general hemorrhage added to the

complications by both mother and child [19].

The symptoms of malaria during pregnancy differ with the intensity of malaria transmission and with the level of immunity acquired by the pregnant women [20]. Since malaria transmission intensity may vary within the same country from areas of relatively stable transmission to areas of unstable or epidemic transmission. The clinical picture of malaria infection during pregnancy may likewise range from asymptomatic to severe life threatening illness. Atypical manifestation of malaria is more common in pregnancy particularly in primigravida. The patient may have the different pattern of fever from a febrile to continuous pyrexia. In the second half of pregnancy, there may be more frequent paroxysm of fever. Patient living in hyperendemic area may present with a severe anaemia without any definite pyrexia. Enlargement of the spleen may be variable in the course of pregnancy [21, 22].

Microscopic examination of blood smears has been the gold standard for malaria diagnosis, but it is compromised by poor infrastructure and the need for individuals with expertise in microscopy who are not necessarily available in many health facilities in malaria endemic regions [23]. Furthermore, microscopy is not sensitive enough [24-29], it requires good quality reagents, well maintained microscope, and is time consuming [30].

There are few prospective studies assessing the effects of malaria during pregnancy in relation to gestational age, especially for vivax malaria [31-42].

Relying on microscopic identification of malaria species jeopardizes malaria control due to its limitations [43, 44]. The WHO recognizes a need for rapid, accurate, and easy diagnostic tool in order to control malaria and need for such a test is mounting in developing countries [45]. Currently available rapid diagnostic tests (RDTs) are controversial due to their sensitivity and specificity and differentiate poorly between plasmodium species [46-48].

With the expanding use of nanotechnology in the biomedical arena, nanoparticles can play a role in low cost, innovative diagnostics [49]. Gold nanoparticles aggregate and change their color from red to purple-blue upon exposure to single stranded DNA in aqueous solution while double stranded DNA stabilize them to preserve red color and thus present an opportunity to develop a fast and easily interpreted diagnostic test [50-55].

Merozoite Surface Protein 10 (MSP10) is an immunogenic protein encoded by a single copy gene (in P.vivax, PVX_114145) which is expressed in the asexual blood stages of it [56].

Rationale

Blood based tests can discourage screening of P.vivax, because of the pain associated with finger prick and because of social and cultural beliefs about blood sampling. Less painful, more culturally sensitive, and safer tools for malaria diagnosis should encourage participation in mass screening programs and improve public health. Urine contains its circulating DNA in detectable quantities and can therefore serve as a less invasive and more acceptable sample for malaria screening and diagnosis. Also, urine contains less interfering proteins and inhibitors than blood which allows easier DNA extraction. Furthermore, urine provides lower risks to healthcare personnel, with reliable amounts of malaria DNA found in urine despite being substantially lower than blood samples. Additionally, urine color is not expected to obscure color change of gold nanoparticles.

To control malaria, there is an urgent need for applying innovative diagnostics and new technologies. Nanoparticles can augment detection of malaria at lower parasite levels while providing fast and simple method. Novel use of MSP10 and gold nanoparticles to identify its DNA in urine can be utilized as screening tool with global application potentials. The proposed test could impact the control of the vivax malaria in low resource settings as it could present a simple, fast, cheap and easy to interpret test. Furthermore, utilizing urine instead of blood eliminates the need for finger-prick which would increase safety profile and likely increase participation rate in mass screening programs.

More over there is no published study about colorimetric detection of pregnancy associated vivax malaria in urine using MSP10 Oligonucleotides and gold nanoparticles.

Research questions

What is the sensitivity and specificity of colorimetric system using MSP10 oligonucleotides and gold Nanoparticles for detection of pregnancy associated vivax malaria in comparison to gold standard method (direct microscopy)?

Is it possible to achieve the research project during the expected time frame (April /2019- June /2021)?

Research hypothesis

In this study, we will test the hypothesis that a colorimetric system using gold Nanoparticles and MSP10 DNA detection in urine will be useful as a safe diagnostic and surveillance tool for it. Such a tool needed for improving malaria diagnosis and control in the endemic settings.

Objectives

General objectives

To introduce new, innovative, safe, rapid and accurate diagnostic method for pregnancy associated vivax malaria using MSP10 oligonucleotides and gold Nanoparticles to save the life of pregnant women and their babies.

Specific objectives

- To compare between sensitivity of colorimetric system using MSP10 oligonucleotides and gold Nanoparticles and microscopy for it from urine and blood specimens
- To compare between specificity of colorimetric system using MSP10 oligonucleotides and gold nanoparticles and microscopy for P.vivax from urine and blood specimens.
- To compare between Plasmodium vivax density obtained by microscopy and color changes through colorimetric method.

Materials and method

Study design:

Case control study

Study area:

The study will be conducted in Khartoum State.

Study population:

- Pregnant women with vivax malaria whom will match the selection criteria

  Selection Criteria:

  Inclusion criteria:

  Pregnant women with of the following criteria:

  • Live in Khartoum State.

  • Free from HIV, syphilis and Hepatitis

  • Free from proteinuria

  • Free from diabetes.

- Not fasting.

  Exclusion criteria:

  Pregnant women with one or more of the following criteria:

  • Live out of Khartoum State.

  • HIV, syphilis and Hepatitis B&C pregnant woman

  • Diabetic pregnant woman.

  • Pregnant woman with proteinuria.

- Fasting.

  Sample size:

200 pregnant women

Case group:

100 pregnant women with vivax malaria

Control group:

100 normal pregnant women.

MSP10 Oligonucleotides:

The two MSP10 oligonucleotides will utilize in this study. Crafted to represent the C-Terminal segment of MSP10, the first oligonucleotide has a sequence of 5´CACCATGGAACAGTTT ATCCTGAAGAC3'. The other oligonucleotide will be used as a representative of the N-terminal segment of MSP10. It has a sequence of 5´AGCCATGGAACGTGCTAAGTGCAACA3' .

Urine Samples:

Urine samples from positive for P.vivax will collect from Iquitos. Negative control urine samples will be collected from volunteers who will be blood smear negative in Iquitos. All urine samples will be collected by clean catch rules. Firstly we will do urine analysis to all specimens then samples match study requirements will be pelleted in the field and shipped on dry ice, pH will adjusted and samples will be refrozen at-80°C.

Blood samples:

5 ml of venous blood samples will collect under antiseptic condition from both the study and control group. Initially, each blood specimen will be screened for HIV, hepatitis and syphilis, and then specimens match the study requirements will used for further analytical processes.

Blood smears:

During the study, we shall report whether a slide is positive or negative and will identifies the species (only P.vivax will be included).We shall read 100 fields before the slide reported as negative.

In the laboratory, we will start a second read to the slide to report species and parasite density according to the white blood cell count of each participant.

Parasite densities (parasite/µl of whole blood) will be calculated as follows: (Number of parasites counted/WBCs counted) X WBCs count/µl of blood.

For quality control, 10% randomly selected slides (positive and negative) will be reexamined by two blinded, expert microscopists at a national reference laboratory.

Colorimetric system:

Urine will thaw at 25°C.Once urine being at room temperature, dipsticks will carried out to determine urine pH and presence of protein. .Each urine sample will be centrifuge at 15,000 rpm for 5 minutes to remove sediments and then filtered using a 0.2mm membrane filter to remove possible confounding particulates. The urine samples will be diluted1:16 with PBS. Diluted samples' pH will be adjusted to reach 6.4 using pH meter, and HCl and NaOH solutions. 50 uL of each diluted urine sample will be heated at 95°C for30 seconds using a thermocycler. Samples will be cooled at room temperature for 10 minutes and 10uL of either C-Terminal or N-Terminal MSP10 oligonucleotides and 20uL of 0.25MNaCl will add. The sample will heated at 59°C for two minutes and allowed to cool to room temperature for ten minutes. Finally, 50uL of citrate reduced AuNPs will be added. Two minutes later, the system will be read visually and by spectrophotometer.

Ethical approval:

Urine and blood samples will collect for the study after being approved by research ethical committee at Alneelain University. Written informed consents will obtain before storing samples as anonymous and unidentified.

Data collection:

Patients data will collected through well constructed questionnaire contain: Name, date of birth, gender, address, occupation, telephone number, and result of study mandatory tests (HIV, hepatitis, syphilis, blood glucose, and urine analysis, CBC).

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{false Negative}}$$

$$\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{false positive}}$$

Statistical analysis:

The results will be analyzed using SPSS, version 16.0 for Windows (SPSS Inc, Chicago, IL, USA, 1989-2007).

Work plan

Activity	April-October/2019	November-July/2020	August-December/2020	January-May2020	June-December/2020	January-June /2021
Proposal development and submission						
Collection of urine and blood specimens						
Laboratory analysis						
Data analysis and report writing						
Write up thesis						
Write up papers						

Facilities required

1. Light microscope
2. Bench centrifuge
3. Thermal cycler
4. Hematological analyzer
5. Spectrophotometer.
6. PH meter
7. Automatic pipette
8. Refrigerator -80 C
9. HCG urine strips
10. ICT kits for HIV
11. ICT kits for hepatitis B & C
12. ICT kits for syphilis
13. Urine strips comber10
14. Glucose reagent
15. Plain containers
16. EDTA containers
17. Giemsa powder 3.8 gm
18. Glycerol 250 ml
19. Absolute Methanol 250 ml
20. 70% Methanol
21. 5% Sodium hypochlorite
22. Glass slides
23. Cover glass
24. Pasteur pipettes
25. Staining rack
26. Centrifuge tubes
27. Gauze
28. Cotton
29. Urine containers
30. Eppendrof tubes
31. 5ml Syringes
32. Citrate reduced gold15nm nanoparticles
33. Potassium chloride
34. Phosphate buffer saline
35. Sodium chloride
36. Hydrochloric acid

37. Sodium hydroxide
38. TwoMSP10oligonucleotides
39. Pencils
40. Markers
41. Gloves

Budget

Number	Item	Quantity	Price
1	ICT for HIV	8 boxes	**180 USD**
2	ICT for HBV	8 boxes	**15 USD**
3	ICT for HCV	8 boxes	**30 USD**
4	ICT for Syphilis	8 boxes	**24 USD**
5	HCG urine strips	4 boxes	**1.17**
6	Urine strips comber 10	3 boxes	**1.5 USD**
7	Glucose reagent	I vial (500ml)	**10 USD**
8	Giemsa powder	3.8 grams	**21.28 USD**
9	Glycerol	250 ml	**1.0 USD**
10	Absolute methanol	500 ml	**1.25 USD**
11	Distilled water	5 liters	**1.45 USD**
12	Citrate reduced 15nm gold nanoparticles		**384.45 USD**
13	Potassium chloride	5gm	**3.25USD**
14	Sodium chloride	5 gm	**0.002 USD**
15	Hydrochloric acid	500 ml	**1 USD**
16	Phosphate buffer saline		**2.3 USD**
17	5% Sodium hypochlorite	One litre	**4.6 USD**
18	Sodium hydroxide	500 ml	**0.85 USD**
19	MSP10 oligonucleotides	Two	**100 USD**
20	5ml syringes	3 boxes	**5.9 USD**
21	Plain containers	300 piece	**8.4 USD**
22	EDTA containers	300 piece	**12 USD**
23	Urine containers	300 piece	**3 USD**
24	Eppendrof tubes	300 piece	**2.1 USD**
25	Centrifuge tubes	300 piece	**2.7 USD**
26	Forested glass slides	5 boxes	**2.25 USD**
27	Cover glass	3boxes	**7.5USD**
28	Pasteur pipettes	500 piece	**0.5 USD**
29	Staining rack	2 racks	**1.4 USD**
30	Cotton	3 rolls	**3.531 USD**
31	Gloves	3 boxes	**13.5 USD**
32	Gauze	2 rolls	**2 USD**
33	Pencils	3 piece	**0.12 USD**
34	Markers	3 piece	**0.6 USD**

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