Research proposal

Comparative detection of Plasmodium vivax DNA in saliva, urine and blood samples from plasmodium vivax malaria patients in Sudan

By

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2018

Introduction and literature review

Malaria is the most widespread infectious illness in the tropics and subtropics (1). Currently *P.vivax* is endemic across Asia, the south pacific, North Africa , Middle East , South and central America(2), and has lately recurred in regions where it had formerly been eliminated, including North America and Europe (3). .presently, an estimated 2.9 billion people live at risk of P.vivax infection (4). Research in malaria has been principally focused on P.falciparum, the most lethal form of malaria. However, P.vivax can also cause severe sickness with serious complications and expenses, particularly in children, in whom it has a major impact on growth (5– 7).

Presently accessible diagnostic techniques for malaria include the identification of malaria parasites or parasite proteins in blood by microscopy and rapid diagnostic tests (RDT) and parasite DNA by polymerase chain reaction (PCR). Microscopic examination of Giemsa-stained thick blood film is the diagnostic gold standard, but even qualified microscopists habitually detect only 50–100 parasites or more/microlitre of peripheral blood (8, 9), therefore missing infected individuals with very low parasitaemia (10,11). People existing in low malaria transmission areas harbor submicroscopic parasitaemia (12, 13) and represent reservoirs that protract transmission (14). Immunochromatographic tests detect malaria parasite-specific proteins, such as histidine-rich protein-2 (HRP-2) in plasma. RDTs are effortless to achieve and fitting for field settings. Although multi-survey analyses prove a strong association between prevalence of malaria by RDT and by microscopy (15), RDTs detect more infections than microscopic inspection (8, 10, and 16). Furthermore HRP-2-based RDTs may show false positive results for up to a month following parasite clearance (11, 16-18). Molecular methods are highly sensitive with PCR detecting as low as 1–5 parasites/ µl of blood (19-21, 1). although differences in their procedure s and performances, microscopy, RDT, and nested PCR (nPCR) for malaria diagnosis share a common problem, that is, the requisite of blood samples. Collection of blood is not risk-free. Pain is experienced and phobias associated with finger pricks and needles have been clinically explained (22-24). In addition, cultural and religious myths discourage some persons from providing blood samples. These aspects decrease contributor compliance in epidemiological surveys where frequent sampling is required. Besides, accidental

transmission of blood borne pathogens such as HIV and hepatitis viruses can occur during blood draws and finger-pricks. Less painful, more culturally sensitive, and safer tools for malaria diagnosis should support participation in mass screening programs and improve public health(25) and better technique for malaria diagnosis is required that incorporates the accuracy of PCR and the use of non-invasive clinical samples.

Human being saliva is readily obtainable and is increasingly being recognized as an important diagnostic specimen. While more than 98% of human saliva is composed of water (26), the salivary fluid holds many constitutive and infiltrating electrolytes, proteins, and DNA that have been explored for diagnoses and screening of a broad range of illnesses(27-29). such as, molecular markers of malignancy, for example p53 mutations and carcinoembryonic antigen have been detected in saliva of patients with oral carcinomas (30) ,and saliva concentrations of some biomarkers of cardiovascular illness correlate with equivalent serum concentrations (31). In addition, saliva-based kits have been developed for the diagnosis of HIV (32) and human papilloma virus (33). Although some researchers have also detected malaria parasite DNA in urine samples, the sensitivity of saliva-based detection is significantly higher than urine-based detection of malaria parasite DNA (34-38). Therefore, saliva is a more promising non-blood substitute for malaria diagnosis than urine.

Study done by Pattakorn Buppan et al showed that; microscopy results, nested PCR of saliva samples had a sensitivity of 84% for P. vivax detection. Both nested PCR result of saliva samples had a specificity of 100% for identification of P. vivax when compared with nested PCR results from blood. Although the positive rates of nested PCR of saliva samples for P. falciparum increased with parasite density, no tendency occurred in results from nested PCR of saliva samples for P. vivax (39).

Rationale

Existing malaria diagnostic methods require blood collection that may be associated with pain and the risk of transmitting blood-borne pathogens, and often create poor compliance when repeated sampling is required. On the other hand, the collection of saliva and/or urine is minimally invasive; but those specimens have not been widely used for the diagnosis of malaria. So we want to alternate the conventional methods with modern and noninvasive techniques.

Also it is necessary to identify sub-microscopic in order to reduce transmission. However, some aspects of monitoring and evaluation, post-treatment follow-up and long-term epidemiological studies require frequent blood sampling. This has resulted in patient dissatisfaction, and children and pregnant women are sometimes, unlikely to participate in these invasive investigations. Ideally, sampling should be simple, non-invasive, reliable and painless. In recent years, malaria has been diagnosed by sampling of saliva and urine, and this may feasibly replace blood sampling. The aim of this research is to validate the use of human saliva and urine for nested PCR-based vivax malaria detection among selected group of Sudanese individuals.

Objectives

General objectives

To compare the diagnostic performance of saliva, urine and blood specimens for diagnosing Plasmodium vivax malaria with conventional method (microscopy).

Specific objectives

1. To compare between the sensitivity and specificity of nested PCR of saliva samples and microscopy for P.vivax.
2. To compare between the sensitivity and specificity of nested PCR of urine and microscopy for P.vivax.
3. To compare between the sensitivity and specificity of nested PCR of blood and microscopy for P.vivax.

Materials and method

Study design: Prospective, comparative hospital based - study.

Study period: study will be conducted from November 2018 to June 2020.

Sample size: 100 of saliva, urine and blood specimens will be collected from each participant.

Study population:

Inclusion criteria: untreated vivax malaria patients positive by microscopy of any age group that will be seen at Basher teaching hospital, Soba University hospital and Ibrahim malik teaching hospital who will be willing to participate.

Exclusion criteria: Individuals having previous anti-malarial treatment and those with bleeding gum.

Ethical consideration

Informed consent will be obtained from the participants. A good discussion will be conducted with them to explain the benefits of the research for the community and any possible out come from the research will be accounted for their benefits.

Sample collection and storage

We will collect 2 ml of blood (in a sterile tube containing EDTA), 2 ml of saliva and 5 ml of urine, both in 2 volumes of absolute ethanol. All samples will be stored at -20C until analysis.

Malaria microscopy

Both thin and thick blood smears will be prepared for each isolate and will be stained with 10% Giemsa solution. The thin blood film will be examined for at least 200 microscopic fields and the thick blood film for at least 200 leukocytes, using a 100× objective. Parasitaemia (parasites/µl of

blood) will be determined by counting the number of parasites against 200 white blood cells and multiplying a constant of 8000 white blood cells/µl of blood. Each slide will read by two experienced microscopists and their parasitaemia readings will averaged. Discrepancies greater than 10% between two readings will be resolved by the reading of a third technician.

DNA extraction

. DNA will be extracted from200 ml of whole blood using the QIAamp DNA Mini Kit (QIAGEN Ltd, Hilden, Germany) following manufacturer's instructions. Ethanol in the saliva and urine samples was removed by centrifuging at 18500 g for 3 min, washing the sediment three times with sterile PBS and suspending in 200 ml PBS. DNA from saliva samples was extracted using the QIAamp DNA Mini Kit (QIAGEN Ltd) using the protocol of Verweij et al (40). While DNA from urine samples was extracted using the QIAamp viral RNA kit (QIAGENLtd) to provide a higher DNA yields (41).

Nested PCR assay

The mitochondrial cytochrome b gene of P.vivax will be targeted by primers in a nested PCR reaction, using conditions described by Putapurntip et al. (35) .Primers will be designed to amplify a 227 bp fragment in the first PCR reaction. This fragment was then diluted in sterile distilled water and used as a template in the second PCR reaction. Species-specific primers will be added in separate reaction tubes to amplify a181 bp P.vivax fragment in the second PCR reaction (35).The amplified products were visualised on 2% agarose gels stained with ethidium bromide.

The genomic DNA was extracted from all of the blood samples using Agencourt Genfind v2 DNA isolation kit (Beckman Coulter, Beverly, Massachusetts, USA) according to the manufacturers 'protocol. This DNA templates were used for the amplification with thermal cycler (Sensoquest, Go_ttingen, Germany) using a genus specific primer set as described by Perandin et al. (42). In order to assess P. vivax, a species-specific primer set was used (43) All of the PCR reaction mixtures (100 ml of total volume) consisted of 250 mM deoxynucleoside triphosphate (Fermantas RO191), 10 × PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 20 mM MgCl2), 2 U/ml Taq DNA polymerase (Fermantas SB38) and 5 pmol of each primer set. The thermal cycling began with denaturation at 95°Cfor 5 min, followed by 25 cycles of 94°C

for 30 sec, 58℃ for 2 min, and 72℃ for 2 min, with final incubation at 72℃for 5 min. Five-ml aliquots of the product were used for the second amplification cycle (nested 2) using the same PCR conditions and thermal cycle except the use of 30 cycles instead of 25. PCR products were analyzed on agarose gels of 2.5% by electrophoresis at 100 V in 1 ×Tris-Boric-EDTA buffer (0.04 M Tris-boric and 1 mM EDTA pH 8.0) and then visualized by UV light after being stained with ethidium bromide.

Statistical analysis

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

Work plan

	10-11/2018	**12/2018-5/2019**	**6-10/2019**	**11-12/2019**	**1-3/2020**	**4-6/2020**			
Proposal development and submission									
Collection of saliva , urine and blood specimens									
Laboratory analysis									
Data analysis and report writing									
Write up thesis									
Write up papers									

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