ORIGINAL ARTICLE

PREVENTIVE MEASURES AND DIAGNOSIS OF ASYMPTOMATIC MALARIA IN NORTHWEST ETHIOPIA

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ABSTRACT

***Background**: Asymptomatic malaria poses a huge challenge in meeting the malaria, elimination goal. Due to the poor sensitivity of the common laboratory diagnostic methods of malaria like rapid diagnosis tests (RDTs) and Giemsa stained microscopy, it has often been difficult to detect asymptomatic malaria. Thus, highly sensitive, specific, and field deployable rapid molecular techniques need to be available to accurately detect the disease. This study aimed to assess the application of malaria preventive methods and to assess the prevalence of asymptomatic malaria, using the traditional Giemsa microscopy and molecular methods, such as loop mediated isothermal amplification (LAMP) and nested PCR in the study sites.*

***Methods**: A community based cross-sectional study was conducted from February to May 2014 in North Gondar, Ethiopia. A total of 802 participants were enrolled. Data on socio-demographic profile and associated risk factors for asymptomatic malaria were collected using face-to-face interviews. Capillary blood was collected and blood films and dried blood spots (DBS) were prepared for malaria parasite detection using microscopy, nested polymerase chain reaction (nPCR), and loop-mediated isothermal amplification (LAMP).*

***Results**: In this study, 45.3% of the participants had access to combined universal preventive measures of malaria. LAMP and nPCR were performed for 160 DBS samples. The overall prevalence of asymptomatic malaria detected using Giemsa microscopy, LAMP, and nPCR was 3.75%, 4.375% and 4.375%, respectively.*

***Conclusion**: A consistent use of the available malaria prevention methods was too limited to produce the intended elimination of malaria in the study area. LAMP was able to identify two extra asymptomatic malaria carriers per 100 study population. This study indicated that an active diagnosis of asymptomatic malaria with molecular techniques, like LAMP, could support malaria elimination through enhanced active case detection. Future studies should evaluate the performance of LAMP and nPCR from fresh blood samples.*

***Key words**: Asymptomatic malaria; Malaria Elimination; LAMP; Ethiopia.*

BACKGROUND

Over the last decade, the world has moved forward in the fight against malaria by the implementation of universal intervention measures, such as mass distribution of insecticide-treated bed nets (ITNs), an increased use of long-lasting insecticide-treated nets (LLIN), early diagnosis, and prompt use of artemisinin-based combination therapy (ACT). As a result, the burden of malaria has been significantly reduced globally [1]. However, the latest report of the World Health Organization (WHO) revealed that in 2015 alone there were 214 million cases of malaria and 438,000 malaria deaths globally. Ninety percent of the deaths occurred in sub-Saharan Africa [1]. In Ethiopia, malaria is one of the major public health problems and an obstacle to socio-economic devel-

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opment [2]. In 2012/13, there were 57,503 public sector malaria hospitalizations and 4,984,266 malaria outpatient cases. Of the total malaria outpatient cases, only 84.27% were confirmed positive by laboratory diagnosis [3].

Additional interventions, such as active and proper detection of asymptomatic *Plasmodium* parasite carriers need to be addressed, especially where malaria elimination programs are launched [4]. Although asymptomatic malaria carriers could not usually present symptoms of fever, rigors, and chills, they account only for 20-50% of all human-to-anopheles mosquito transmissions [5, 6]. Ethiopia has targeted to achieve 50 malaria-free districts by 2020. However, active surveillance of asymptomatic malaria is not included in the program [7]. Earlier studies in Ethiopia revealed a prevalence of 19.2% sub-microscopic *Plasmodium falciparum* carriage [8] and 6.8% of asymptomatic malaria [9] when detected by PCR and Giemsa microscopy, respectively.

Asymptomatic malaria parasite carriers are common in endemic areas and could represent an important reservoir for malaria transmission. Many of these individuals can carry microscopically detectable levels, while others can carry sub-microscopic levels of *Plasmodium* parasitemia [10]. The use of clinical diagnostic methods, such as rapid diagnosis tests (RDTs) and Giemsa microscopy leads to underestimation of the burden of asymptomatic malaria [11].

As a result, highly sensitive molecular methods such as polymerase chain reaction (PCR) remain to be the essential diagnostic methods for the detection of submicroscopic malaria parasites [12, 13]. However, the high cost of PCR makes it less applicable to malaria mass screening programs in resource-limited communities. Therefore, field deployable and user friendly rapid molecular tools, such as loop medicated isothermal amplification (LAMP) are highly needed to replace PCR to support malaria control and preventive programs. LAMP is designed to detect genus *Plasmodium* down to species level with a limit of detection of as low as 1-10 parasites/µl of whole blood within an hour of total turnaround time [14, 15].

Therefore, this pilot study aimed to determine the prevalence of asymptomatic malaria using microscopy, LAMP, and nPCR, in North Gondar, Ethiopia.

METHOD

Study design, period, and area: A community based cross-sectional study was conducted from February to May 2014 in four districts of North Gondar, namely Gondar Zuria, Dembia, Tacharmachiho, and Metema, Amhara Regional State, Ethiopia, (**Figure 1**). According to the 2008 Central Statistical Agency (CSA) report, North Gondar has a total population of 2,929,628 in an area of 45,934.090 square km and a population density of 64 persons per square km.

The districts of Dembia, Gondar Zuria, Metema, and Tacharmachiho account for 315,903, 278,835, 83,000 and 89,115 of the total population and 1,295, 1,215, 3,995, and 8,456 square Kilometers of the land mass, respectively. According to the zonal municipality report, these areas are historically believed to be malaria endemic, and malaria is among the top three public health problems in the area.

Study subjects: Non-febrile individuals who had lived at least for one year in the study areas and aged

above 18 years were enrolled in the study. Individuals who were taking anti-malarial treatments in the two weeks preceding the screening were excluded. In this study, asymptomatic malaria is defined as the presence of *Plasmodium* infection in the absence of clinical signs and symptoms of malaria, like fever, chills, and rigors at enrollment [11].

Sample size and study subject selection: A total of 802 apparently healthy individuals from the four sites were recruited into the study. We selected these sites because they have been malaria endemic with low malaria transmissions. From each study site, one kebele (sub-district) was randomly selected, and participants were recruited at village level until the 200 target was reached. Investigators together with village health extension workers notified the community of the mass screening for asymptomatic malaria at the local health post.

After the aim of the study was explained, participants who gathered at the health post were requested to take part in the study voluntarily. Then, volunteers reported to the sample collection point and recruited. Individuals with overt clinical signs and symptoms of malaria were excluded. Socio-demographic data, such as age and gender, as well as use of malaria prevention measures were collected, using an interview-based questionnaire.

Blood sample collection and processing: Capillary blood samples were collected aseptically from finger pricks, using sterile blood lancets. The first drop of blood was removed and four consecutive drops were taken to prepare the thick and thin blood films. Moreover, two separately placed dried blood spots (DBS) in Whatman filter paper 903 (GE Healthcare) labeled with subject identification numbers were prepared. The blood films and DBS were allowed to air-dry at room temperature. Thin blood films were fixed with a 99.5% methanol and kept in slide boxes, while the DBS samples were placed in sealable plastic bags with desiccant and transported to the University of Gondar. The DBS samples were then shipped to the University of Calgary, Canada for LAMP and PCR analysis.

Microscopic detection of malaria parasites: Blood films were stained with 10% Giemsa-stain solution for 10 minutes within 24 hours of field collection. Then, the slides were examined for detection and species identification of *Plasmodium* parasites by two malaria microscopists at the University of Gondar. One hundred 100X microscopic fields were examined to determine the presence or absence of *Plasmodium* parasites.

Nested polymerase chain reaction (nPCR): One hundred sixty (160) DBS samples (40 from each study site) were selected consecutively for nPCR and LAMP analysis. Genomic DNA was extracted from dried blood on Whatman filter paper 903 (GE Healthcare), following previously published protocol [16]. Briefly, two six millimeter diameter blood containing filter paper confetti were cut from each sample using a sterile paper puncher, and genomic DNA was extracted using Quick-gDNA™ Blood MiniPrep kit (Zymo research corp., USA).

The procedure in the company instructions manual was followed with a slight modification. Briefly, the blood spot confetti were treated with 200µl of genomic lysis buffer for 30 minutes with quick vortex every five minutes. The supernatant was then transferred into a Zymo-Spin™ IIC Column in a collection tube, washed with wash buffer, and eluted with 40µl of sterile distilled water.

Nested PCR was performed, following a previously published procedures with a slight modification [16-18]. The Small subunit ribosomal RNA gene was amplified using genus-specific primers (rPLU5 and rPLU6). Then, the PCR product was diluted 1:5 in sterile distilled water. The second PCR was done from the diluted PCR product in three separate tubes, using species-specific primers, rFAL1/rFAL2, rVIV1/rVIV2, rOVA1/OVA2, for *P. falciparum*, *P. vivax*, and *P. ovale*, respectively.

Loop-mediated isothermal amplification (LAMP): Loopamp^{TM} malaria Pan/Pf detection kit (Eiken Chemicals, Tokyo, Japan) consisting of plastic reaction tubes, containing thermo-stable vacuum-dried reagents were used to amplify *Plasmodium/ P. falciparum* DNA. Loop-mediated isothermal amplification (LAMP) was performed, using a non-instrumented nucleic acid amplification (NINA) device (PATH, USA). The NINA heater device was used to produce isothermal conditions suitable for DNA amplification (63-65 ^{o}C). The LAMP reaction produced turbidity which was detected visually as a result of the accumulation of a large quantity of insoluble magnesium orthophosphate, a byproduct of DNA amplification. The preparation of the LAMP mixture, running, and detection was performed as described previously [16].

Data analysis: Data was entered into Microsoft Excel (2007) and transferred into SPSS version 20 (SPSS Inc. Chicago, 2007) software for statistical analysis.

Ethical considerations: Ethical clearance was obtained from both research and ethics committees of the University of Gondar (CMHS/08/281/2013) and the University of Calgary (REB16-0720). After explaining the objective and relevance of the study, permission was also secured from each district health office. In addition, after explaining the benefit of the study, verbal informed consent was obtained from each study participant. *Plasmodium* positive cases were communicated to the respective local health extension worker for appropriate treatment according to the national guideline.

RESULT

Demographic and malaria indices of the population: A total of 802 study participants were enrolled in the study, of whom 44.4% were males and 55.6% females. The mean ($\pm$SD) age of the participants was 33.5 ($\pm$15.4) years. Among the study population, 5.1% were not treated with anti-malaria drugs for their previous malaria episodes. Although 45.3% of the study population used combined malaria intervention measures, most of them were using either of the alternative prevention and control measures of malaria. Fifty percent of the population were using long lasting impregnated bed nets. Furthermore, 46% of the participants always slept under bed nets. On the other hand, 50.7% had access to insecticide residual spray (IRS); the practice was exercised among only 31.7% of the participants.

Magnitude of asymptomatic malaria diagnosed using Giemsa microscopy, LAMP, and Npcr: The prevalence of asymptomatic malaria was 3.75% (6/160), 4.37% (7/160) and 4.37% (7/160) when detected by Giemsa microscopy, LAMP and nPCR, respectively. LAMP was able to identify one mixed infections of *P. falciparum* and pan-malaria (genus-specific *Plasmodium*), however, neither nPCR nor Giemsa microscopy identified any mixed infection.

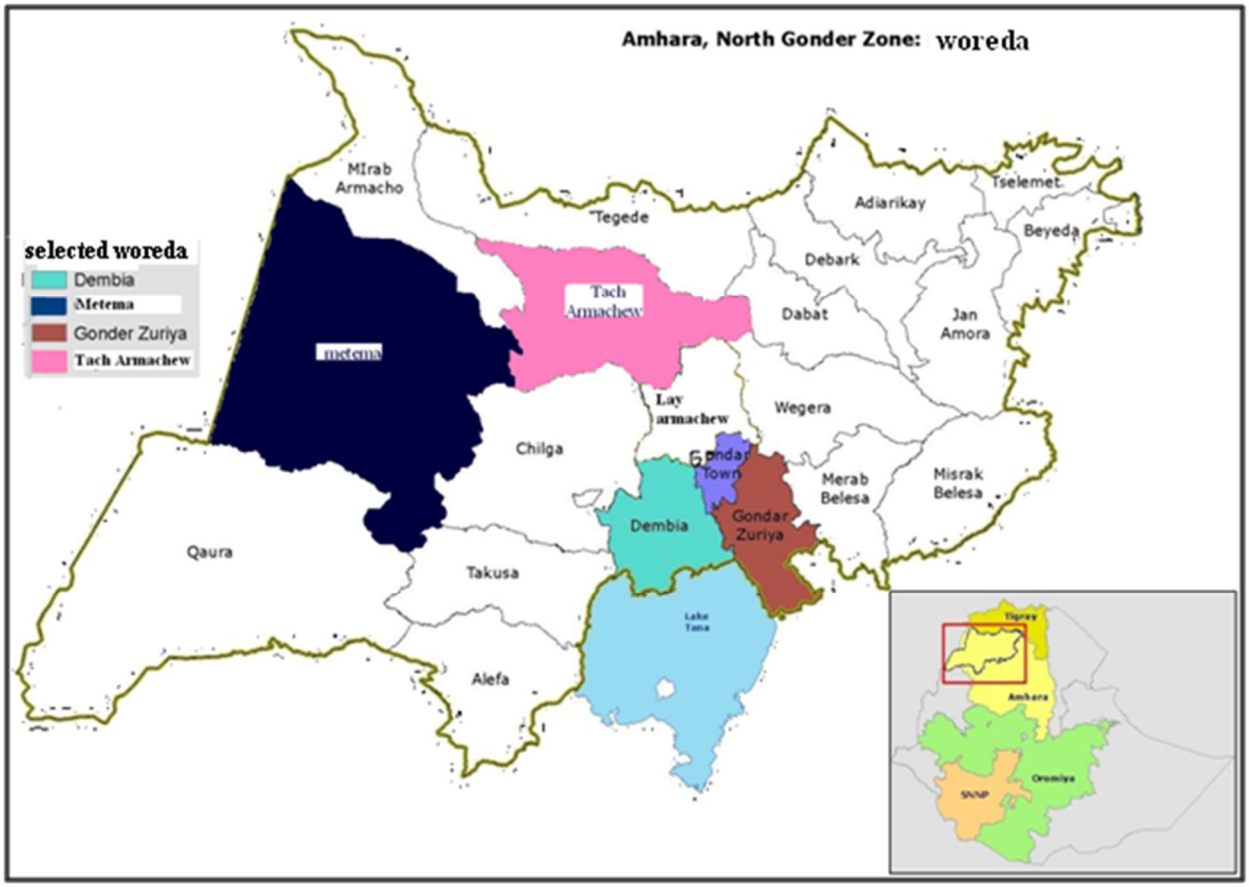

Figure1: Map of study districts in North Gondar Zone, Amhara Regional State, Ethiopia.

DISCUSSION

When the level of clinical malaria decreases due to a consistent implementation of universal intervention measures, the rate of low density malaria parasite carriers gets higher [5]. This could account for an estimated transmission source of 20-50% of all human-to-anopheles mosquito transmissions [6]. Therefore, the detection of asymptomatic malaria parasite in historically malaria transmission endemic countries plays a significant role in malaria elimination endeavors [4, 19].As a result, the need for a high throughput user friendly rapid detection technique for malaria mass screening is necessary. Nested polymerase chain reaction is considered as a sensitive and reliable detection method compared to other routinely available methods, Giemsa microscopy and RDTs [12]. However, nPCR is well adapted to laboratory settings and is not affordable to make it accessible in community-based malaria mass screening and treatment programs. Thus, a more effective, affordable and field-deployable method is required to detect asymptomatic malaria in mass screening programs. Studies have demonstrated that LAMP is a better alternative to nPCR [15, 20]. These studies revealed that with a minimal training and time of processing, LAMP could provide a comparable result to nPCR for the detection of low density malaria parasitaemia [15, 20].

We have previously shown that LAMP is a cost effective implementable method in resource limited settings, and more sensitive than RDTs and micros-

copy for the detection of malaria parasites in febrile malaria suspected patients at health facility levels. In this study, we have found that LAMP was comparable to nPCR and superior to Giemsa microscopy for the detection of both *Plasmodium* genus and *P. falciparum* species [16].

In this study, we have found equivalent prevalence of asymptomatic malaria by LAMP and nPCR, 4.37%. In an earlier study in northwest Ethiopia, a Giemsa microscopy based prevalence of asymptomatic malaria parasite of 6.8% among school age children was higher than that of the present study [9]. This could be due to the difference in the data collection period. The present study was carried out during the dry season when the transmission of asymptomatic malaria to immune population was low.

Among Giemsa microscopy positives, 16.7% (1/6) of the samples were detected positive by nPCR, whereas, among LAMP positives, 42.9% (3/7) of the samples were positive by nPCR. There were four discordant results between LAMP and nPCR where such (4) samples tested positive by LAMP were negative by nPCR and vice versa. None of the microscopically positive samples were detected by LAMP. In addition to the inherent limitations of Giemsa microscopy, subjectivity and misdiagnosis [23] could be higher than in molecular techniques [24]. The relative detection limit of Giemsa microscopy is about 20 parasites/µl of whole blood [25].

The present study is similar to studies in Zanzibar [15, 20] that LAMP could potentially support the evaluation of the impact of improved access to universal intervention measures through an increased detection of low density malaria parasite carriers in communities. A simulation study also showed that community mass screening with sensitive diagnostic techniques and treatment of cases would play a more significant role than universal intervention measures alone in accelerating malaria control to elimination levels [27]. One thing to be considered is the actual coverage of universal intervention measures of communities and the practical use of these interventions at household levels. The present study revealed gaps in access to and practice of these interventions in the study area. Similarly, a study in Nepal showed that access to universal intervention measures still needs improvement [28].

CONCLUSION

Access to malaria prevention methods and a consistent use of the available methods is too limited to bring the intended result of malaria elimination in the study area. Therefore, more effort should be exerted to maximize access to the prevention methods and to increase the use of already deployed preventive measures. LAMP is able to identify two extra asymptomatic malaria carriers per 100 in our population. This study suggested an active diagnosis of asymptomatic malaria using molecular methods, such as LAMP will aid the fight against malaria through improved detections of low density malaria parasites. Future studies on the evaluation of LAMP and nPCR as diagnostic methods in fresh blood samples are recommended. Molecular diagnostic methods were done only on samples collected from 160 of the 802 participants in the study. This is because of the high cost of running LAMP. Thus, it was not possible to evaluate the diagnostic performance of the tests using measurements of sensitivity and specificity.

Authors' contribution: SG, AAF, LW and DRP

conceived the study. SG, LW, GG, MB, YW, GF, ME, AAF, BM and AA participated in field data collection. AA contributed in the diagnosis of malaria parasites with Giemsa microscopy. AGB carried out LAMP and nPCR analysis. SG computed the statistical analysis. SG, AGB and DRP drafted the manuscript. All authors contributed to and approved the final submitted manuscript.

Competing interest: The authors declare they have no any issue that might impose competing interest.

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