Research Square

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Expansion of the *Plasmodium falciparum* Kelch 13 R622I mutation in Northwest Ethiopia

Aberham A. Alemayehu
University of Gondar College of Medicine and Health Sciences
Daniel Castaneda-Mogollon
University of Calgary Cumming School of Medicine
Habtie Tesfa
University of Gondar College of Medicine and Health Sciences
Sisay Getie
University of Gondar College of Medicine and Health Sciences
Abu Naser Mohon
University of Calgary Cumming School of Medicine
Nirujah Balasingam
University of Calgary Cumming School of Medicine
Abebe G. Bayih
Armauer Hansen Research Institute
Dylan Pillai (drpillai@ucalgary.ca)
University of Calgary Cumming School of Medicine

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Abstract

According to the WHO, almost two thirds of the Ethiopian population are at risk of contracting malaria, where infection with Plasmodium falciparum accounts for approximately 60% of cases today. The risk of artemisinin resistance spreading from SE Asia to Africa is a major concern. We conducted a 28-day *in vivo* efficacy trial of Artemether-Lumefantrine (Co-Artem) for treatment of uncomplicated malaria (n = 97) in the Gondar Region, North West Ethiopia in 2017–2018. Our results confirmed 100% adequate clinical and parasitological response (ACPR) with no parasites observed at day 3 by microscopy. Further analysis of day 0 samples showed the expansion of a kelch13 mutation R622I to 9.5% from 2.4% of isolates reported three years earlier. Closer examination of the R622I mutation *in vitro* is warranted.

Introduction

Malaria remains as one of the top leading causes of morbidity and mortality among low-income countries, infecting 229 million people and 409,000 deaths according to the latest World Health Organization (WHO) report (1). Despite global efforts, these numbers have only decreased modestly for the past five years (1). Reasons underlying this include a higher number of *hrp2/3* deletions in *Plasmodium falciparum* that decreases the sensitivity in rapid diagnostic tests (RDTs); high level of detection (LOD) diagnostic tools for mass screen and treatment (MSAT) campaigns; neglect of asymptomatic cases; increased prevalence of insecticide-resistance *Anopheles* mosquitoes; geographical expansion of artemisinin-resistance clones, among others (1–7). The latter poses a challenge to the effectiveness of artemisinin-based combination therapies (ACT), which is the first-line treatment for uncomplicated *Plasmodium falciparum* malaria (8–10).

Ethiopia, with a current population of over 110 million is a malaria-endemic country with an estimate of 68% of its population at risk of contracting the disease (1). The main vectors of transmission in the area are *A. funestus, A. arabiensis*, and *A. pharaoensis* which are known to be highly seasonal and predominant below an altitude of 2,500 meters (11). Unlike most of Sub-Saharan countries where malaria is almost exclusive to *P. falciparum*, Ethiopia displays a relatively high number of *Plasmodium vivax* cases, making it the fourth country with the highest incidence for this strain in 2018 (1). Despite this, Ethiopia has shown a remarkable decrease of malaria cases through public health interventions using prevention and control of malaria among pregnant women by intermittent preventive treatment (IPTp), vector control through indoor residual spraying (IRS), an increase of insecticide treated nets (ITNs) availability, among others (1, 12).

The presence of artemisinin-resistant *P. falciparum* strains was reported for the first time in 2006(13, 14) at the Thai-Cambodia border, and eventually expanded across southeast Asia Although some cases of artemisinin resistance were found Guyana and Papua New Guinea, there is no clear evidence of ACT failure due to parasite resistance in the African continent. Additionally, *Plasmodium falciparum* artemisinin-resistant clones have been previously correlated to copy number variation (CNV) of the *plasmepsin2/3* genes, along with single nucleotide polymorphisms (SNPs) of the *Pfcoronin*(15),

pfFd(16), and *arps10*(16). Nevertheless, the aforementioned genetic polymorphisms could hardly be connected with clinical artemisinin resistance at the field level. Instead, multiple SNPs in the *Kelch13* gene including C580Y, F446I, Y493H, R539T, I543T, P553L, R561H, P574L, A675V, R622I, M579I, have been strongly associated with clinical artemisinin resistance (17–19). Previously it was thought that *Kelch13* mutants were exclusively found in Viet Nam, Thailand, Cambodia, Guyana, and Papua New Guinea. Nevertheless, recent studies have identified mutants in various African countries, with a considerable prevalence of the A578 mutation (20, 21). Some studies have shown *in vitro* resistant strains in Rwanda, not affecting the overall efficacy of dihydroartemisinin/piperaquine (DP) or artemether/lumefantrine (AL) (17). According to the Worldwide Antimalarial Resistance Network (WWARN) database, more than 15 countries have reported an artemisinin success rate below 90% across a 28-day drug efficacy trial between 1977 and 2017 (22). While this is not direct evidence of *Kelch13* mutants conferring resistance, this data should be taken into consideration when designing public interventions for malaria treatment among these countries. Previous studies have shown how the *Kelch13* protein compartment and its interactome play a role in resistance through a decreased rate of hemoglobin endocytosis, which reduces the by-product hemoglobin concentration needed for artemisinin activation (23). Recent studies have shown evidence of *in vivo* delayed parasite clearance and *in vitro* artemisinin resistance through ring-stage assays (RSA) of various *Kelch13* SNPs.

An *in vivo* ACT efficacy trial study was conducted in the Gondar region of notrthwest Ethiopia in 2014 (Fig. 1), where the presence of the R622I SNP in the *Kelch13* propeller domain was first documented (19). In this study, we have conducted a follow-up study in the same geographical area in 2018, applying a similar methodology described in 2014.

Methods

This study was reviewed and approved by University of Calgary (Ethics ID 15-3204) and University of Gondar (Ethics ID: CMHS/4571/08). The study was conducted at two health centers (Negade Bahir and Dembia General Hospital) in northwest Ethiopia between 2017 and 2018. A total of 97 patients were enrolled in a 28-day efficacy trial of artemether-lumefantrine (Co-Artem) as per Ethiopian Ministry of Health guidelines. All patients cleared the infection by day 28. Peripheral blood was collected for malaria diagnosis, along with DNA extraction for the *Kelch13* gene sequencing. Mono-infected patients were included in the study Finger-pricked samples were collected on a Whatman 903 paper filter (GE Healthcare, Mississauga, Canada) at the time of the diagnosis, air-dried, and stored in zip-lock bags for its transportation to the University of Calgary, Canada. Genomic DNA extraction was performed with the QIAamp DNA Mini kit and by following the manufacturer's protocol of DNA isolation from blood samples (24). The *Kelch13* gene was amplified using nested polymerase chain reaction (nPCR) from previous protocols (19). Capillary sequencing was then performed at the Centre of Health Genomics and Informatics (CHGI) at the University of Calgary using Applied Biosystems (Burlington, ON, Canada) 3730 XL 96 capillary DNA analyzer. Multiple sequence alignment (MSA) was done using Clustal Omega software (25) across all of the isolates with the wild type (WT) *Kelch13* reference gene (Genbank:

MK877455.1), and edited manually to remove any existing gaps through Jalview (26). Sequenced reads with a low degree of alignment or “Ns” present at the codon of interest were excluded of the analysis.

Results

In this study we have identified an increase in prevalence of the R622I mutant in the northwest Ethiopian region close to the Sudan border. This mutation was previously reported in the same region in 2016 (19). This mutant has been recorded by Chinese imported cases between 2016 to 2018 (27), along with isolates from Eritrea (28), and Somalia (29). Capillary sequencing was performed bidirectionally across all the isolates. The MSA file showed a high degree of similarity among the propeller domain gene (supplementary file 1). Five DNA reads were excluded from the analysis due presence of “N” in the codon of interest or due a low degree of alignment with the rest of the sequences.

Demographic and epidemiological variables of interest were collected at the time of diagnosis (Table 1). A total of 86 isolates could be sequenced from 97 dried blood spots collected at day 0 of the diagnosis. Samples prior to treatment were used for this study. Capillary sequencing and MSA was obtained across all of the 86 isolates sequenced, 78 (90.7%) were indicative of the expected wild type arginine at the 622nd position, while the remaining 8 samples (9.3%) showed a consistent isoleucine mutation in both the forward and reverse reads (Table 2). Only one isolate presented a synonymous “CGA” mutation in the forward read and the expected WT “AGA” codon in the reverse read.

Table 1
Epidemiological and clinical features of the study participants. ACPR – adequate clinical and parasitological response.

Epidemiological variable (n)	n (%)
Age (97)	
Under 5	2 (2.06)
Between 6–15	37 (38.14)
Adults	58 (59.79)
Gender (97)	
Female	37 (38.14)
Male	60 (61.86)
Fever (97)	
No	4 (4.12)
Yes	93 (95.88)
ACPR (97)	
Yes	100 (100)

Table 2
Non-synonymous and synonymous K13 mutants at two sites in the Gondar region of North West Ethiopia. GH- General Hospital.

Collection site	Samples collected (n)	Samples sequenced (n)	K13 synonymous mutations (n)	R622I K13 non-synonymous mutations (n)
Negade Bahir	71	66	2	4
Dembia GH	26	20	0	4

The reported R622I mutant prevalence is considerably higher when compared to the 2.4% reported in 2016 (19) (3.88 fold increase), considering the relatively small time frame of four years between sample collection. Besides the R622I, one of the analyzed isolates had the V510V synonymous mutation ("GTG" to "GTA") that has been previously reported in southern Nigeria in 2018 (30). None of the isolates presented any of the mutations that have been reported in the Greater Mekong Subregion (F446I, Y493H, R539T, I543T, P553L, R561H, P574L, C580Y, A675V) (31, 32)

Discussion

In this study we have identified an increase in prevalence of the R622I mutant in the northwest Ethiopian region close to the Sudan border. This mutation was previously reported in the same locale in 2016 but at a lower frequency (19). This mutant has been recorded imported cases to China between 2016 to 2018 (27), along with isolates from Eritrea (28), and Somalia (29). Our data clearly demonstrates an increase of this mutant in northwest Ethiopia, although no association with *in vivo* artemisinin resistance could be established. Several reasons may have contributed to this clonal expansion of this SNP containing parasites in the study area. First, the R662I SNP could potentially provide the parasite with a biological advantage in the presence of artemisinin but has not been correlated to *in vivo* parasite clearance studies potentially due to a higher naturally acquired immunity (NAI) among this study population; a minor proportion of the immunocompromised people may have acted as the reservoir for the spread of the R622I SNP containing parasites. Second, the R662I SNP probably has a role to support elevated *in vivo* fitness of the parasite. Third, these mutant parasites do not possess the necessary background mutations to exert in vivo resistance against ACT.

Some of the limitations of the study include capillary sequencing instead of next-generation sequencing (NGS). By doing this, we were not able to determine if the non-synonymous mutations present were from the dominant *Plasmodium falciparum* clone in each patient. Moreover, the isolates that displayed the wild type arginine may harbor any minority variant clones with mutations as capillary sequencing typically captures the information of the most predominant clone. The small sample size also may skew the true prevalence of this mutation.

While no *in vivo* parasite-delayed clearance has been correlated to *Kelch13* SNPs in Africa, molecular surveillance of artemisinin resistance genes should remain operational in order to identify any changes in treatment phenotypes. Of note, a recent report of the R561H kelch13 mutant in Rwanda suggested clonal expansion of this mutant and correlation to *in vitro* resistance (17). To date, there are no *in vitro* studies correlating the R622I codon to parasite resistance, and while this study does not provide direct evidence of a new treatment-tolerant parasite, CRISPR/Cas9 RSA studies should be conducted to determine the biological significance of this mutant given its clonal expansion and spread to other regions over a relatively short period.

Abbreviations

ACT
Artemisinin combination therapy
SNP
Single nucleotide polymorphism
WHO
World Health Organization
hrp2/3
Histidine rich protein 2/3
RDT
Rapid diagnostic test
LOD
Level of detection
IPTp
Intermittent preventive treatment among pregnant women
MSAT
Massive screening and treatment
IRS
Indoor residual spraying
ITN
Insecticide treated net
CNV
Copy number variation
DP
Dihydroartemisinin/piperaquine
AL
Artemether/lumefantrine
WWARN
Worldwide antimalarial resistance network
RSA
Ring-stage assay
nPCR
nested polymerase chain reaction
CHGI
Centre of Health Genomics and Informatics
MSA
Multiple sequence alignment
WT
Wild type
NAI
Naturally acquired immunity

NGS
Next generation sequencing

Declarations

Ethics approval and consent to participate

This study was reviewed and approved by University of Calgary (Ethics ID 15-3204) and University of Gondar (Ethics ID: CMHS/4571/08).

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

DRP is an Associate Editor of Malaria Journal

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Authors' contributions

Conceptualization: DRP, AGB; Methodology: DCM, AA, HT, ANM, SSG, NB Formal Analysis: DCM, AA, HT, ANM, SSG, NB, DRP, AGB; Investigation: DCM, AA, HT, ANM, SSG, NB, DRP, AGB ; Data Curation: DCM, AA, HT; Writing – Original Draft: DCM, DRP; Writing – Reviewing & Editing: DCM, AA, HT, ANM, SSG, NB, DRP, AGB; Supervision: AGB, DRP; Funding Acquisition: DRP.

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References

1. World Health Organization. World Malaria Report. 2020. Geneva.
2. Cheaveau J, Mogollon DC, Mohon MAN, Golassa L, Yewhalaw D, Pillai DR. Asymptomatic malaria in the clinical and public health context. Expert Rev Anti Infect Ther [Internet]. 2019 Dec 2 [cited 2020 Feb 5];17(12):997–1010. Available from: https://doi.org/10.1080/14787210.2019.1693259.

3. Ashley EA, Phyo AP, Woodrow CJ. Malaria. The Lancet [Internet]. 2018 Apr 21 [cited 2020 Nov 19];391(10130):1608–21. Available from: https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(18)30324-6/abstract.
4. Larsen DA, Bennett A, Silumbe K, Hamainza B, Yukich JO, Keating J, et al. Population-Wide Malaria Testing and Treatment with Rapid Diagnostic Tests and Artemether-Lumefantrine in Southern Zambia: A community Randomized Step-Wedge Control Trial Design. Am J Trop Med Hyg [Internet]. 2015 May 6 [cited 2020 Nov 19];92(5):913–21. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4426577/.
5. Cook J, Xu W, Msellem M, Vonk M, Bergström B, Gosling R, et al. Mass Screening and Treatment on the Basis of Results of a Plasmodium falciparum-Specific Rapid Diagnostic Test Did Not Reduce Malaria Incidence in Zanzibar. J Infect Dis [Internet]. 2015 May 1 [cited 2020 Nov 19];211(9):1476–83. Available from: https://academic.oup.com/jid/article/211/9/1476/854482.
6. Verma AK, Bharti PK, Das A. HRP-2 deletion: a hole in the ship of malaria elimination. Lancet Infect Dis [Internet]. 2018 Aug 1 [cited 2020 Nov 19];18(8):826–7. Available from: https://www.thelancet.com/journals/laninf/article/PIIS1473-3099(18)30420-1/abstract.
7. van Eijk AM, Sutton PL, Ramanathapuram L, Sullivan SA, Kanagaraj D, Priya GSL, et al. The burden of submicroscopic and asymptomatic malaria in India revealed from epidemiology studies at three varied transmission sites in India. Sci Rep [Internet]. 2019 Nov 19 [cited 2020 Nov 19];9(1):17095. Available from: https://www.nature.com/articles/s41598-019-53386-w.
8. Treating malaria [Internet]. [cited 2020 Nov 19]. Available from: https://www.who.int/activities/treating-malaria.
9. Pousibet-Puerto J, Salas-Coronas J, Sánchez-Crespo A, Molina-Arrebola MA, Soriano-Pérez MJ, Giménez-López MJ, et al. Impact of using artemisinin-based combination therapy (ACT) in the treatment of uncomplicated malaria from Plasmodium falciparum in a non-endemic zone. Malar J [Internet]. 2016 Jul 2 [cited 2020 Nov 19];15(1):339. Available from: https://doi.org/10.1186/s12936-016-1408-1.
10. Information NC for Pike B USNL of M 8600 R, MD B, Usa 20894. TREATMENT OF UNCOMPLICATED PLASMODIUM FALCIPARUM MALARIA [Internet]. Guidelines for the Treatment of Malaria. 3rd edition. World Health Organization; 2015 [cited 2020 Nov 19]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK294441/.
11. Ethiopia. Malaria | IAMAT [Internet]. [cited 2020 Nov 22]. Available from: https://www.iamat.org/country/ethiopia/risk/malaria.
12. Girum T, Shumbej T, Shewangizaw M. Burden of malaria in Ethiopia, 2000–2016: findings from the Global Health Estimates 2016. Trop Dis Travel Med Vaccines [Internet]. 2019 Jul 12 [cited 2020 Nov 22];5. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6626392/.
13. Jambou R, Legrand E, Niang M, Khim N, Lim P, Volney B, et al. Resistance of Plasmodium falciparum field isolates to in-vitro artemether and point mutations of the SERCA-type PfATPase6. Lancet Lond Engl. 2005 Dec;3(9501):1960–3. 366(.

14. Vijaykadga S, Rojanawatsirivej C, Cholpol S, Phoungmanee D, Nakavej A, Wongsrichanalai C. In vivo sensitivity monitoring of mefloquine monotherapy and artesunate-mefloquine combinations for the treatment of uncomplicated falciparum malaria in Thailand in 2003. Trop Med Int Health TM IH. 2006 Feb;11(2):211–9.
15. Sharma AI, Demas AR, Hartl DL, Wirth DF. Reply to Velavan et al.: Polymorphisms of pfcoronin in natural populations: Implications for functional significance. Proc Natl Acad Sci [Internet]. 2019 Jun 25 [cited 2020 Nov 18];116(26):12613–4. Available from: https://www.pnas.org/content/116/26/12613.
16. Bwire GM, Ngasala B, Mikomangwa WP, Kilonzi M, Kamuhabwa AAR. Detection of mutations associated with artemisinin resistance at k13-propeller gene and a near complete return of chloroquine susceptible falciparum malaria in Southeast of Tanzania. Sci Rep [Internet]. 2020 Feb 26 [cited 2020 Nov 18];10(1):3500. Available from: https://www.nature.com/articles/s41598-020-60549-7.
17. Uwimana A, Legrand E, Stokes BH, Ndikumana J-LM, Warsame M, Umulisa N, et al. Emergence and clonal expansion of in vitro artemisinin-resistant Plasmodium falciparum kelch13 R561H mutant parasites in Rwanda. Nat Med [Internet]. 2020 Oct [cited 2020 Nov 17];26(10):1602–8. Available from: https://www.nature.com/articles/s41591-020-1005-2.
18. Conrad MD, Rosenthal PJ. Antimalarial drug resistance in Africa: the calm before the storm? Lancet Infect Dis [Internet]. 2019 Oct 1 [cited 2020 Nov 17];19(10):e338–51. Available from: https://www.thelancet.com/journals/laninf/article/PIIS1473-3099(19)30261-0/abstract.
19. Bayih AG, Getnet G, Alemu A, Getie S, Mohon AN, Pillai DR. A Unique Plasmodium falciparum Kelch 13 Gene Mutation in Northwest Ethiopia. Am J Trop Med Hyg [Internet]. 2016 Jan 6 [cited 2019 Dec 23];94(1):132–5. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4710417/.
20. Ménard D, Khim N, Beghain J, Adegnika AA, Shafiul-Alam M, Amodu O, et al. A Worldwide Map of Plasmodium falciparum K13-Propeller Polymorphisms. N Engl J Med. 2016;23(25):2453–64. 374(.
21. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, et al. Spread of Artemisinin Resistance in Plasmodium falciparum Malaria. N Engl J Med [Internet]. 2014 Jul 31 [cited 2020 Nov 27];371(5):411–23. Available from: https://doi.org/10.1056/NEJMoa1314981.
22. Pisani E, Botchway S. Sharing individual patient and parasite-level data through the WorldWide Antimalarial Resistance Network platform: A qualitative case study. Wellcome Open Res [Internet]. 2017 Aug 16 [cited 2020 Nov 23];2. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5627501/.
23. Birnbaum J, Scharf S, Schmidt S, Jonscher E, Hoeijmakers WAM, Flemming S, et al. A Kelch13-defined endocytosis pathway mediates artemisinin resistance in malaria parasites. Science. 2020;03(6473):51–9. 367(.
24. QIAamp DNA, Mini Kit. - QIAGEN Online Shop [Internet]. [cited 2020 Nov 23]. Available from: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/qiaamp-dna-mini-kit/#orderinginformation.

25. Sievers F, Higgins DG. Clustal Omega, accurate alignment of very large numbers of sequences. Methods Mol Biol Clifton NJ. 2014;1079:105–16.
26. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. Jalview Version 2–a multiple sequence alignment editor and analysis workbench. Bioinforma Oxf Engl. 2009 May;25(9)(1):1189–91.
27. Wang X, Ruan W, Zhou S, Huang F, Lu Q, Feng X, et al. Molecular surveillance of Pfcrt and k13 propeller polymorphisms of imported Plasmodium falciparum cases to Zhejiang Province, China between 2016 and 2018. Malar J [Internet]. 2020 Feb 4 [cited 2020 Nov 20];19. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7001319/.
28. L'Episcopia M, Kelley J, Patel D, Schmedes S, Ravishankar S, Menegon M, et al. Targeted deep amplicon sequencing of kelch 13 and cytochrome b in Plasmodium falciparum isolates from an endemic African country using the Malaria Resistance Surveillance (MaRS) protocol. Parasit Vectors [Internet]. 2020 Mar 14 [cited 2020 Nov 20];13. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7071742/.
29. Warsame M, Hassan AM, Hassan AH, Jibril AM, Khim N, Arale AM, et al. High therapeutic efficacy of artemether–lumefantrine and dihydroartemisinin–piperaquine for the treatment of uncomplicated falciparum malaria in Somalia. Malar J [Internet]. 2019 Jul 11 [cited 2020 Nov 20];18. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6624891/.
30. Oboh MA, Ndiaye D, Antony HA, Badiane AS, Singh US, Ali NA, et al. Status of Artemisinin Resistance in Malaria Parasite Plasmodium falciparum from Molecular Analyses of the Kelch13 Gene in Southwestern Nigeria [Internet]. Vol. 2018, BioMed Research International. Hindawi; 2018 [cited 2020 Nov 23]. p. e2305062. Available from: https://www.hindawi.com/journals/bmri/2018/2305062/.
31. Amaratunga C, Andrianaranjaka VH, Ashley E, Bethell D, Björkman A, Bonnington CA, et al. Association of mutations in the Plasmodium falciparum Kelch13 gene (Pf3D7_1343700) with parasite clearance rates after artemisinin-based treatments—a WWARN individual patient data meta-analysis. BMC Med [Internet]. 2019 Jan 17 [cited 2020 Nov 23];17(1):1. Available from: https://doi.org/10.1186/s12916-018-1207-3.
32. Ocan M, Akena D, Nsobya S, Kamya MR, Senono R, Kinengyere AA, et al. K13-propeller gene polymorphisms in Plasmodium falciparum parasite population in malaria affected countries: a systematic review of prevalence and risk factors. Malar J [Internet]. 2019 Mar 7 [cited 2020 Nov 23];18(1):60. Available from: https://doi.org/10.1186/s12936-019-2701-6.
33. NordNordWest. Location map of Ethiopia (1995–2020) [Internet]. Available from: https://creativecommons.org/licenses/by-sa/3.0/de/legalcode.

Supplemental Data

Supplementary file 1 not available with this version

Figures

Figure 1

Map of Ethiopia. The blue star represents the geographical area where the health centres of Negade Bahir and Dembia are located (33). Photo used for map is licensed under CC BY 2.0.