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RESEARCH ARTICLE

Aetiologies of acute undifferentiated febrile illness at the emergency ward of the University of Gondar Hospital, Ethiopia

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Abstract

Objective: Causes of acute febrile illness (AFI) often remain undetermined in developing countries, due to overlap of symptoms and limited available diagnostics. We aimed to assess the aetiology of AFI in adults in a referral hospital in northwest Ethiopia.

Methods: While all participants were tested for malaria by rapid diagnostic test (RDT), microscopy was only done on physician's request. Dengue virus (DENV) infections were detected using an RDT and ELISAs and dengue, yellow fever and chikungunya cases were identified by PCR. Bacterial aetiologies were investigated using blood culture and PCR.

Results: The aetiology of acute infection was identified for 20.5% of 200 patients enrolled. Eleven percent tested positive for *Plasmodium*, while microscopy was only requested for half of the identified malaria cases. For 4.0% of the *Plasmodium*-infected patients, an acute or past DENV (co-)infection was detected. We found 7.5% acute and 13.0% past DENV – all serotype 3 – infections. Bacterial infections were observed in 4.5% of the patients.

Conclusion: Malaria is still a considerable aetiology of AFI and dengue is underrecognised. There are areas where both diseases occur concomitantly, and the DENV-3 serotype presumably spreads from Sudan to northern Ethiopia. As only 20.5% of the aetiologies were identified, a broader testing platform is required.

KEYWORDS
acute febrile illness, aetiology, arbovirus, bacteria, malaria, northwest Ethiopia

INTRODUCTION

Acute undifferentiated febrile illness (AFI) is the most common reason for clinical presentation to health care services in developing countries. It can range from mild, self-limiting to progressive, life-threatening disease. AFI patients present with non-specific symptoms such as fever, headache and malaise, which can be caused by a wide range of pathogens [1].

In the past decade, there has been a shift in importance of pathogens causing AFI. Studies on malaria showed that 80% of febrile illness, even in malaria-endemic regions, are caused by other pathogens, like *Rickettsia*, *Borrelia*, *Leishmania* and arboviruses [2–5]. Moreover, the decline of

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malaria in sub-Saharan Africa due to control strategies puts a greater focus on non-malarial AFI [6].

The clinical symptoms of malaria, arboviral and bacterial infections resemble each other, which makes it difficult to differentiate them clinically [7]. In addition, reliable diagnostic tests for many infections are often not available in hospitals or even in national reference laboratories in resource-limited settings [8]. In Ethiopia in particular, health institutions do not provide full laboratory services to diagnose AFIs [9]. Consequently, when patients present with AFI, the requested laboratory tests are limited to complete blood cell count, urinalysis and microscopy and/or rapid diagnostic tests (RDT) for malaria. The capacity of microbiology laboratories in most areas is also limited or absent. Accordingly, clinical management depends mainly on knowledge of the predominant local aetiologic pathogens. AFIs are often treated empirically with antibiotics and/or antimalarials [10]. Therefore, local data on the aetiology of AFI are urgently required.

In this study, we investigated the aetiologies of undifferentiated acute fever in patients presenting at the emergency ward of University of Gondar Comprehensive and Specialized Hospital (UoGCSH) in northwest Ethiopia. We used 12 tests to identify malaria, bacteria and the most expected arboviral diseases to gather preliminary evidence of these targeted pathogens in northern Ethiopia. This will increase the awareness on circulating infectious diseases among clinicians and aid management of AFI patients.

METHODS

Study design, site and participants

A cross-sectional study was conducted at the emergency ward of UoGCSH in Amhara region in northwest Ethiopia as described before [11]. The ward has 50 beds, serving a population of about 7 million. The Immunology and Molecular Biology laboratory of the UoG has capacity for qPCR and ELISA, while routine Giemsa staining, microscopy and blood culture facilities are available at the Emergency and Microbiology Laboratories of the UoGCSH.

AFI patients (defined by an axillary temperature of ≥37.5°C and symptom duration ≤7 days) who were ≥15 years old and seeking health care in the emergency ward were eligible for recruitment. To focus on undifferentiated fever, febrile patients suspected by the treating physician to have urinary or respiratory tract infections were excluded. In total, 200 patients were enrolled.

Sample collection and processing

Study-related sample collection was performed after routine clinical work-up as described previously [11] and consequent procedures are shown in Figure 1. All samples were tested by RDT, ELISA and PCR (study procedures), malaria microscopy (routine procedure) was only performed for a selection of patients, when requested by the treating physician. All laboratory tests were performed by trained staff at UoGCSH in Ethiopia.

Laboratory tests for malaria

Malaria microscopy

Routine microscopic examination of Giemsa stained slides was requested by the physician for 35 (17.5%) patients. Microscopic examination of thick and thin blood films was done by one reader according to WHO guidelines [12], but without parasite grading.

Malaria rapid diagnostic test

All participants were tested with the SD BIOLINE Malaria Ag P.f/Pan rapid diagnostic test (RDT; Abbott Rapid Diagnostics, Zaventem, Belgium) for *Plasmodium* detection. The RDT contains a membrane strip, pre-coated with mouse monoclonal antibodies targeting histidine-rich protein II (HRP-II) specific for *P. falciparum*, and lactate dehydrogenase (panLDH), which is common in all *Plasmodium* species. Test procedures were performed as described in the manual. A HRP-II line together with a pLDH line indicated an infection with *P. falciparum* or a mixed infection with *P. falciparum* and one or more of the other *Plasmodium* species. A single HRP-II line refers to an infection with *P. falciparum*, whereas a single pLDH line indicates infection with one or more of the other *Plasmodium* species.

Laboratory tests for arboviruses

Dengue RDT

The SD BIOLINE Dengue Duo NS1 Ag and IgG/IgM test (Abbott Rapid Diagnostics) is a rapid, *in-vitro* immunochromatographic test designed for the detection of dengue virus (DENV, all four serotypes) NS1 antigen (Ag) and differential IgM and IgG antibodies to DENV on two test strips in one cassette device. Briefly, 100 µl serum was added for NS1 Ag detection and 10 µl serum was used for IgM and IgG detection and the test was conducted according to the manufacturer's instructions.

Dengue IgM and IgG ELISA

All samples were tested with both the IgM Capture DxSelect and IgG DxSelect (Focus Diagnostics, Cypress) indirect ELISA kits. The assays used 10 µL serum each and were performed according to the manual instructions for detection of antibodies to DENV (all four serotypes) confirming

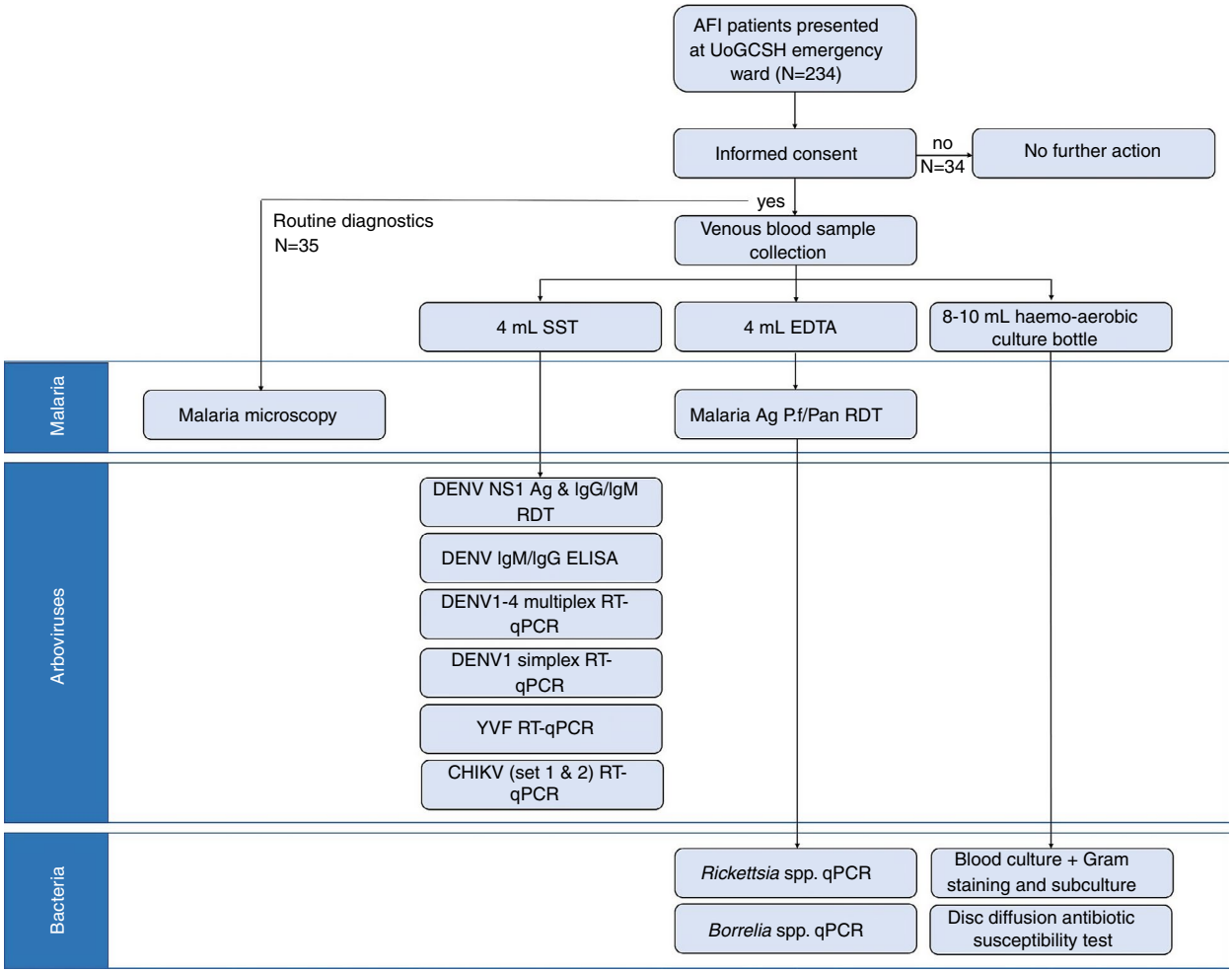

FIGURE 1 Flowchart of methods used to collect and test samples for malaria, arboviruses and bacteria. AFI, acute febrile illness; UoGCSH, University of Gondar Comprehensive Specialized Hospital; SST, serum separator tubes; EDTA, Ethylenediaminetetraacetic acid; RDT, rapid diagnostic test; DENV, dengue virus; YFV, yellow fever virus; CHIKV, chikungunya virus; ELISA, enzyme-linked immune sorbent assay; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction

recent (IgM) and recurrent/past (IgG) infections. The primary antibody was incubated with inactivated DENV1-4 Ag followed by an enzyme-conjugated secondary antibody and substrate. The resultant colour change was quantified by optical density (OD), which was compared with reference cut-off OD readings to determine results for both assays. A background subtraction procedure was included for IgM ELISA as described in the manual to avoid false reactions due to heterophilic antibody activity that could bind directly to the reporter reagent. The ELISA IgM or IgG result was interpreted as positive if the index value was ≥1.

RT-qPCR for dengue, chikungunya and yellow fever

RNA was extracted from 300 μL serum with the addition of 10 μL of phocine distemper virus (PDV) as internal control (IC) using a Maxwell 16 Viral Total Nucleic Acid Purification Kit (Promega, Leiden, the Netherlands) according to the manufacturer's protocol on the Maxwell 16 Instrument (AS1000, Promega). The RNA was subjected to four different assays: *(i)* a multiplex DENV1-4 RT-qPCR [13,14] *(ii)* a simplex DENV1 RT-qPCR [14], *(iii)* a yellow fever virus (YFV) RT-qPCR [15,16] and *(iv)* a chikungunya virus set 1 & set 2 (CHIKV) RT-qPCR [17]. Additionally, samples were subjected to a RT-qPCR targeting PDV to assess the RNA extraction efficiency and check for inhibition [18]. Commercial positive DENV, YFV or CHIKV and negative (nuclease free water) PCR controls were used in each run. A detailed description of the reaction and cycling conditions is presented in Table S1. All assays were run on the QuantStudio 5 real-time PCR instrument (Applied Biosystems, ThermoFisher Scientific). Samples positive with the DENV multiplex RT-qPCR at UoG were retested at the ITM (Belgium).

DENV case definition

An acute DENV infection was defined as a positive DENV RT-qPCR and/or positive IgM ELISA and/or positive NS1 RDT. A past DENV infection was defined as a positive IgG

ELISA with or without IgG RDT in the absence of IgM antibodies, NS1 Ag and DENV RNA. Based on the known low specificity of the IgM/IgG RDT [19], a positive IgM or IgG RDT in the absence of another confirmatory test was not considered a DENV infection and eventually not taken into account as an identified aetiology.

Laboratory tests for bacteria

qPCR for Rickettsia and Borrelia *spp.*

DNA was extracted from 300 μL of EDTA whole blood using the Maxwell 16 LEV Blood DNA kit (Promega) according to the manufacturer's instructions on the Maxwell 16 device (Promega). Extracted DNA was tested for *Rickettsia* spp. of the typhus group (TG) and scrub typhus group (STG) with primers and probes as described by [20] and [21], respectively. The extracts were tested for *Borrelia* spp. with a qPCR based on [22]. All runs were performed on the QuantStudio-5 system (Applied Biosystems). An additional qPCR targeting human haemoglobin-beta (HBB) as described by [23] was performed to check for inhibition and DNA extraction efficiency. A detailed description of the reaction and cycling conditions of the qPCRs is reported in Table S1. Negative (nuclease free water) and positive PCR controls were used in each PCR run.

Blood culture

For blood culture, 8–10 ml of whole blood was collected and added to a Haemoculture bottle (Liofilchem, Roseto delgi Abruzzi, Italy) containing Brain Heart Infusion broth (B.H.I.) with p-Aminobenzoic Acid (PABA) and Sodium Polyanethol Sulphonate (SPS), for aerobic cultivation of microorganisms. Blood culture bottles were incubated at 37°C in a conventional incubator and daily observed for signs of bacterial growth for 7 days. For bottles that showed signs of growth (turbidity, haemolysis and gas production) after 7 days, gram stain and sub-culture were done according to standard procedures for bacterial identification. Skin and environmental bacteria (coagulase-negative staphylococci, *Corynebacterium species*, *Cutibacterium acnes*, *Micrococcus species* and *Bacillus species*) were categorised as blood culture contaminants [24]. Following identification of the bacterial isolates, a disc diffusion method was performed according to Clinical Laboratory Standards Institute (CLSI) 2019 guidelines [25] to test for antibiotic resistance.

Data analysis

Analyses were carried out using SPSS version 26. Descriptive analyses were used to summarize the frequencies and percentages of variables. A Chi-square test (χ^2) was performed between categorical variables (gender, residence) and presence of disease. A p-value ≤0.05 was statistically significant.

Ethics statement

Ethical approval was obtained from the School of Biomedical and Laboratory Science, College of Medicine and Health Sciences, UoG and Institutional Review Board of the Institute of Tropical Medicine (ITM), Belgium with reference numbers SBLS 2123/11 and 1284/19, respectively. Written informed consent was obtained from all participants. The study was registered at www.clinicaltrials.gov (NCT04268732).

RESULTS

Among 200 patients enrolled in the study, the median age was 25 years (IQR 20–35) and 56.5% were males. One hundred and fifty-five (77.5%) participants were living in Gondar town, five (2.5%) in other towns and 40 (20.0%) patients came from rural areas. Clinical data of the participants were described before [11]. Among all AFI cases, the aetiology of the acute infection was identified for 41 (20.5%) patients.

Malaria among AFI patients

In total, 22 of 200 (11.0%) patients tested positive for malaria by microscopy and/or RDT (Figure 2). Seven of 22 patients were positive for non-falciparum *Plasmodium*, either by microscopy only (n = 1), microscopy combined with pLDH positive (n = 3) and pLDH only positive while microscopy was not done (n = 3).

Fifteen patients (7.5%) were infected with *P. falciparum*. Two samples were positive for *P. falciparum* microscopy, HRP-II as well as pLDH (hence, mixed *Plasmodium* spp. infection cannot be excluded). Five were confirmed by microscopy and HRP-II and eight were positive for HRP-II in the absence of a microscopic test.

For half of the malaria cases identified, microscopy was not requested by the treating physician. The prevalence of malaria was higher in males (13.3%) than females (8.0%), although not significantly different (χ^2 = 0.98, p = 0.324). There was no clear difference in the proportion of malaria patients originating from rural (11.3%) and urban (10.0%) areas (χ^2 = 0.015, p = 0.908).

DENV, CHIKV and YFV among AFI patients

Of 200 study participants, 56 (28.0%) were positive for at least one DENV test (Figure 3). In total, 15 (7.5%) acute DENV infections were found by either NS1, IgM ELISA or RT-qPCR. The positive PCR results could not be confirmed at ITM, probably due to low viral load and/or RNA

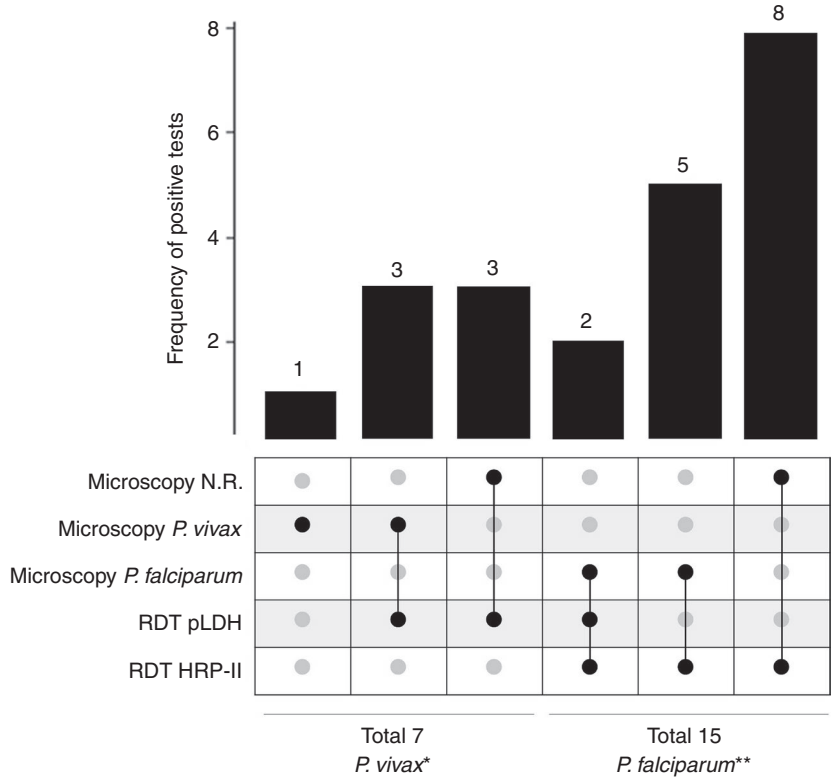

FIGURE 2 Number of patients positive by microscopy and/or malaria RDT and the *Plasmodium* species causing the infection. pLDH is the antigen that is presented on all *Plasmodium* spp., HRP-II Ag is specific for *P. falciparum*. N.R. = Not Requested by physician as routine test. *for three of the 7 *P. vivax* patients, microscopy was not requested to confirm the species. **for two patients, a mixed *Plasmodium* spp. infection could not be excluded as pLDH is not species specific. Microscopy was not requested for 8/15 *P. falciparum* positive cases

degradation. However, PCR contamination cannot be excluded, although the negative PCR control was always negative and dengue viruses were never investigated before in the UoG lab. Cycle threshold (Ct)-values were indeed generally high (>36.5), except for one sample (Ct 27.7) (Figure S1) that was confirmed by IgM and IgG RDT but not by ELISA. All PCR controls confirmed that the implemented assay worked properly and with no previous or ongoing DENV studies at the UoGCSH, hence cross-contamination is very unlikely. All RT-qPCR positive samples were DENV-3 serotype.

Furthermore, 26 (13.0%) patients had a past DENV infection, as determined by IgG ELISA. Fifteen of the IgM and/or IgG RDT positives were not confirmed by ELISA, NS1 or RT-qPCR, and these patients were not considered as DENV infected.

Among 41 acute and past DENV infections, the prevalence was higher ($p < 0.001$) in males (23.9%) than females (16.1%). The positivity rate was similar among patients coming from rural (20.0%) and urban (20.6%) areas. None of the patients were positive for CHIKV or YFV.

Bacteriological profiles among AFI patients

Growth of Gram-positive and -negative bacteria was observed in 8 (4.0%) blood culture bottles, of which one (*Micrococcus sp.*) was considered a contaminant [26]. Antibiotic resistance profiles of the clinically significant isolates are presented in Table S2. *Staphylococcus aureus* grew in four blood culture bottles and was identified as the predominant bacterial agent causing AFI. None of the *S. aureus* isolates showed resistance to methicillin (MRSA). The *Enterobacterales* isolates (two *Klebsiella pneumoniae* and one *Enterobacter cloacae*) showed no resistance to aminoglycosides, ciprofloxacin, piperacillin-tazobactam or meropenem but were all resistant to trimethoprim-sulphamethoxazole. In addition, both *K. pneumoniae* isolates were susceptible to ceftriaxone. By qPCR, we detected two (1.0%) TG *Rickettsia* cases (Ct 25.7 and 38.1), whereas no STG *Rickettsia* or *Borrelia* spp. were found.

Single and mixed infections

The total number of aetiologies found was 46 in 41 AFI patients (Figure 4), not taking the past dengue ($n = 26$ IgG positives) into account. Seventeen patients (41.5%) had malaria only, 11 (26.8%) acute DENV only, and 8 (19.5%) a bacterial infection only. In 5 patients, AFI was caused by a co-infection of malaria and acute dengue ($n = 4$; 9.8%) or malaria and a bacterial infection ($n = 1$; 2.4%).

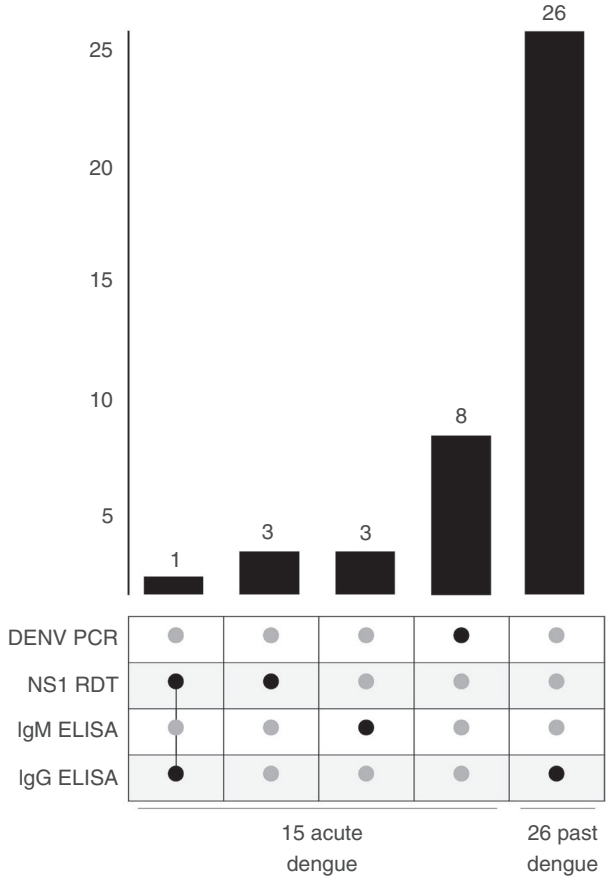

FIGURE 3 Number of patients positive by different tests for DENV and final case definition

DISCUSSION

Attributing a pathogen to the clinical diagnosis of AFI is a hurdle in developing countries, leaving many patients undiagnosed and causing frequent empirical treatment and undetermined causes of death. In this research, we aimed to address the aetiology of AFI in 200 patients by testing them for malaria, several suspected prevalent arboviral and bacterial infections, with a confirmed diagnosis of acute disease for 41 patients.

The proportion of *Plasmodium*-infected individuals in our study was small compared to previous AFI studies conducted in 2009 on children and 2011 on adults in areas at similar altitudes in northern Ethiopia, where about half of the AFI patients had malaria, predominantly caused by *P. falciparum* [9,27]. Our findings were consistent with the result reported by Alemu et al. in 2011 from a health centre near to Gondar town, where 11.5% of 384 fever patients had malaria [28]. That study showed, in contrast, that most infections were *P. vivax*, which is probably because the study was performed in a rural area, while most of the study participants in our study were from urban areas where *P. vivax* patterns are generally lower [29]. Although the non-falciparum *Plasmodium* infections are most probably *P. vivax*, it could also be *P. ovale*, which was found in low numbers in the lowlands in northern Ethiopia [28].

The number of malaria patients identified in our study emphasises that malaria caused by different *Plasmodium* species is still a considerable public health problem. All RDT positives were also microscopy positive (if microscopy was done) and vice versa except one sample, which was *P. vivax* identified by microscopy, but negative by RDT, which could be due to the low parasitemia of the non-falciparum *Plasmodium* infection [30]. For half of the malaria patients, the physician did not suspect malaria and did not request a microscopy test, mainly because Gondar town is not well known as an endemic region. This demonstrates the importance of the physician's decision to request a microscopy test to diagnose malaria patients. Moreover, RDT is a useful tool in settings where microscopy is not available.

Four of the malaria cases in our study were also positive for acute DENV infections, suggesting that there are potentially areas with a burden of both diseases. However, of these, two malaria dengue co-infections were based on the IgM DENV ELISA results. Malaria has been reported to cause 37% false positivity rate in the DxSelect ELISA test, which should be taken into account [31]. A few other studies reported concurrent malaria and dengue disease in countries with high prevalence of both diseases [32–34]. However, once malaria is diagnosed, there is no further suspicion of a potential DENV co-infection, leading to underreporting of dengue. There are recent reports of the emergence of *Anopheles stephensi*, a new malaria vector, which occurs in areas where *Aedes* vectors are common in Ethiopia [35]. Hence, the concomitant burden of the two diseases should be closely monitored and vector integrated interventions should be implemented to tackle both vectors simultaneously.

Due to effective interventions, malaria transmission declined in different regions of Ethiopia, including the Amhara region [36,37], causing the need to look for other causes of AFI. The fact that almost a quarter of the patients in our study (2019) tested positive for a DENV test reflects the endemicity of dengue in Ethiopia, where increasing outbreaks are reported. In particular, DENV has emerged in lowland areas, causing outbreaks in Dire Dawa (2013) [38], Shabelle (2018) and Gode zones (Somalia region, 2014–2016) [39,40], and there was serological evidence for dengue in southern Ethiopia (2016) [41], and Humera and Metema (Amhara region) [42]. Therefore, it is important that physicians include dengue as a differential diagnosis for AFI.

All PCR-positive DENV infections in our study were serotype 3. Previous outbreaks investigations in Dire Dawa and Somali regions in eastern Ethiopia only found serotype 2 [38,40]. Serotype 3 is common in Sudan, which is bordering northwest Ethiopia, indicating that it may have spread from Sudan to Ethiopia, due to human mobility and/or distribution of the vector [43].

In studies conducted in northwest Ethiopia [42], the number of dengue cases was larger in urban than rural areas, as urbanisation facilitates the increase of *Aedes* mosquitoes [44]. We could not find this association, conceivably because there were far less patients recruited from rural areas.

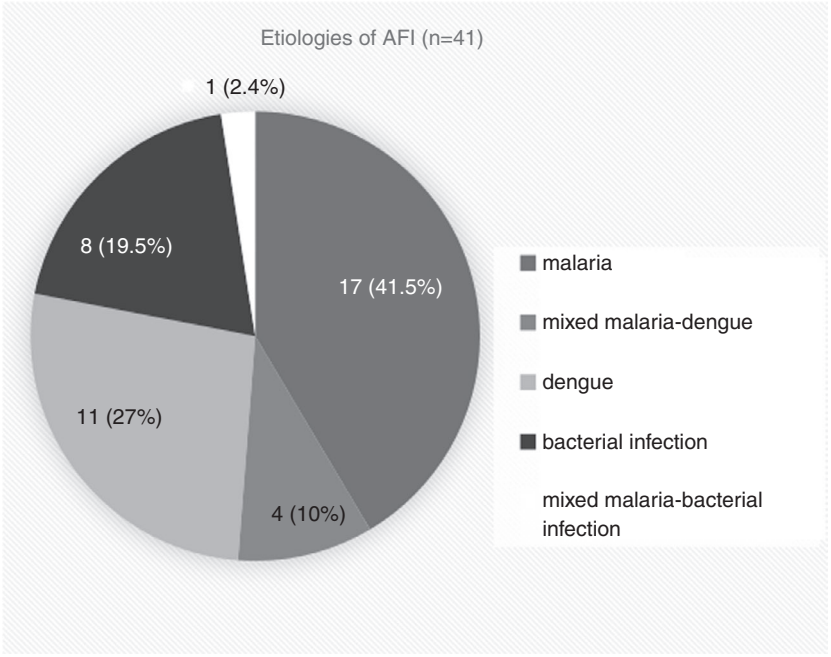

FIGURE 4 Overview of single and mixed infections. % represents the percentage of identified infections on the total number of AFI patients in which an etiology was identified (n = 41). Dengue stands only for acute DENV infections

More male patients had dengue, which agrees with other studies done in northwest Ethiopia [42] and Somali region [40]. Although the travel history of these patients is unavailable, we hypothesise that these patients presumably got infected during a travel to lowland areas, as there is no evidence of *Aedes* mosquitoes at mid-highland altitudes like Gondar. It is well known that substantial numbers of male migrant workers move from Gondar to Metema and Humera at the Ethiopia-Sudan border for labour, where they are plausibly exposed to *Aedes* mosquitoes [42,45]. This indicates that there should be sensibilisation of migrant workers to inform them on how to protect themselves against mosquito bites.

Due to the cross-sectional nature of our study, no follow-up samples were collected and tested for DENV seroconversion, which could further have supported the DENV test results that did not complement each other as one would expect. However, viral RNA can be detected without detection of antigen and vice versa [46,47] and, each of the three test methods individually confirmed acute DENV infections by antigen, RNA or antibody detection along with past DENV infections. Also, viral neutralization testing was not performed and thus other flaviviruses cannot be excluded for the DENV ELISA positive results [6].

Despite recent outbreaks of CHIKV in the Somali and Afar regions in 2019 [48,49] and the re-emergence of YFV in the South Omo Zone [50], we found no chikungunya and yellow fever cases. Yet, there is recent (2017) serological evidence of CHIKV circulation in northwest Ethiopia [51], so we still consider them as potential aetiologies of AFI.

Bacterial infections were previously found considerably high in sub-Sahara Africa [9,52–56]. The low prevalence of bacteremia in our study was presumably due to the suboptimal blood volume sampled (8–10 ml, ethically acceptable for critically ill patients), whereas CLSI guidelines recommend 40–60 ml [25]. Bacterial infections other than bloodstream infections may still be an important cause of acute fever; no tests were done to exclude localized bacterial infections. No important resistance to antimicrobial agents was found, maybe partly due to exclusion of patients who took antibiotics during the last 2 weeks.

Borrelia was not detected and *Rickettsia* pathogens contributed only to a few of the AFI cases in our study, yet previous reports in Ethiopia are indicating that these pathogens are circulating widespread in the country [57–60]. As these atypical bacteria have severe clinical implications, they should still be considered as a differential diagnosis.

Although we included tests for the commonly known circulating pathogens, the aetiology of AFI remained undetermined in 79.5% of the patients. Yet, diseases like visceral leishmaniasis, brucellosis, leptospirosis and acute schistosomiasis, which occur in Ethiopia [61–64], were not tested for in this study. Overall, the large number of unidentified aetiologies indicates the need for implementation of a broader diagnostic platform such as metagenomics [65–67] to increase the knowledge on the circulating pathogens causing AFI, which can be used to improve patient care.

CONCLUSION

Our study is the first in Ethiopia to identify the aetiologies of AFI by testing for malaria, bacteria and specific arboviral diseases altogether. Malaria is still an important cause of AFI and the physician's request for a malaria test is crucial to appropriately manage these patients. DENV is underreported and should be included as an important aetiology. DENV serotype-3 has presumably spread across the Ethiopia-Sudan border. The

small number of bacterial infections is probably an underestimation, but shows that most blood stream infections are caused by *S. aureus*. Overall, this study gathered preliminary evidence on some of the key aetiologies of AFI in northwest Ethiopia, although the remaining large number of undiagnosed AFI calls for the implementation of a broader testing platform.

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SUPPORTING INFORMATION

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