

## Distribution of Dengue Virus Serotype Based on Neutralization Assay in Northwest Ethiopia

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### Abstract

**Background:** Dengue virus (DENV) is arthropod-borne virus transmitted by the bite of *Aedes* mosquitoes. Neighboring countries are highly endemic for DENV, but specific data on the occurrence of DENV in Ethiopia are not available. Therefore, this study aimed to provide information on the presence of different DENV serotypes in the study areas.

**Method:** The study was conducted from January 2016 to March 2017 in northwest Ethiopia. Blood samples were collected from febrile patients and screened for IgM and IgG antibodies against DENV by ELISA. Further, IgM positive samples were screened for dengue NS1 antigen and RT-PCR targeting the NS5 gene of DENV while IgG positive samples were analyzed using neutralization test (ND<sub>50</sub>) to measure titers of dengue serotype-specific antibodies.

**Result:** Out of 114 anti-dengue IgM positive samples, dengue NS1 antigen was positive in 17 (14.9%) while none of the samples were positive for RT-PCR. Neutralization test results were obtained from randomly selected 60 dengue IgG positive samples, and of which 49 (81.7%) had neutralized one or more DENV serotypes. Out of 49 neutralization test positive samples, 27 (55.1%) had neutralizing antibodies to DENV-1, 38 (77.6%) to DENV-2 and 18 (36.7%) to DENV-3. DENV-4 neutralizing antibodies were not detected. The most frequent monotypic neutralizing antibodies were against DENV-2.

**Conclusion:** Dengue virus neutralizing antibodies present in most of the cases investigated. This indicates the presence of dengue infections as neglected cases of fever and thus highlight the importance of dengue surveillance, prevention, and control in the country.

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## Background

Dengue virus (DENV) is an enveloped, single-stranded positive-sense RNA virus within the family Flaviviridae and the genus *Flavivirus* (1). The DENV consists of four closely related, but genetically distinct antigenic serotypes (DENV-1, DENV-2, DENV-3, and DENV-4); a few years ago, a new viral serotype (DENV-5) was reported in Malaysia (2). During a lifetime, persons are susceptible to any of the different DENV serotypes which facilitate the intensity of viral circulation and the occurrence of epidemics (3).

Primary infection with any one of the four DENV serotypes usually remains asymptomatic though it may cause a wide variety of diseases ranging in severity, from mild self-limiting disease, to dengue fever (DF). Particularly, repeat heterotypic infection may cause the more severe dengue hemorrhagic fever/ dengue shock syndrome (DHF/DSS) and death (4). Currently, it is regrouped under non-severe dengue with or without warning signs and severe dengue (5). Recovery from homotypic infection confers lifelong immunity against that particular infecting serotype. However, subsequent infection by other serotypes has been recognized as the main risk for the occurrence of more severe forms of the infection, which can be life-threatening without proper early interventions (6). Subsequent infection with other serotype resulting exaggerated cytokine response leads to tissue damage causing hemorrhage, thrombocytopenia, leucopenia, plasma leakage from capillaries and multi-organ dysfunction and eventually death (7).

Dengue virus is the most important human arthropod-borne virus (arbovirus) transmitted by day-biting mosquitoes of the genus *Aedes*, especially by *Ae. aegypti* and *Ae. albopictus*. The geographic distribution of its infection has expanded to tropical and subtropical countries, and it occurs mostly in urban areas where the vectors are widely found. It has become endemic in more than 100 countries across the Americas, the Eastern Mediterranean, Western Pacific, Africa and South-East Asia (8). According to the World Health Organization, over 2.5 billion people are at risk of acquiring dengue infection, with an estimated 390 million cases per year. Many factors, including rapid uncontrolled urbanization, modulating climatic factors, expansion of mosquito vectors in urban areas, and increasingly widespread international travel and transport are thought to be responsible factors in facilitating the spread of DENV (9).

In Ethiopia, the first documented dengue outbreak occurred in 2013 in the Dire Dawa city with DENV serotype 2 (10). No systematic study has been carried out in other regions of Ethiopia and serotype distribution is unknown. Due to the lack of a single diagnostic assay that can accurately detect dengue infection throughout its clinical course, different diagnostic assays have been developed depending on the duration of the clinical disease. The NS1 antigen ELISA test and RT-PCR have allowed early detection of DENV during short-lived viremia. Serological assays (detection of IgM and IgG antibody) are used at a later time points as well as for study of DENV epidemiology (11).

The dengue NS1 antigen is a non-structural protein produced during viral replication that provides clinicians with a useful tool for diagnosis of acute phases (the first day after the onset of fever up to 9 days) of DENV infections (12, 13). The NS1 antigen detection has become an important diagnostic tool for diagnosing of acute DENV infection in samples in which IgM is not detectable and where PCR is not available. Because of the high specificity of the assay, there is no cross-reaction of dengue NS1 antigen with the other flaviviruses; hence the dengue NS1 antigen ELISA may also be useful as differential diagnostics between flaviviruses (14, 15). The RT-PCR technique has been used to determine the genetic variability of each serotype, the origin, and spread of the viruses during epidemics and outbreak clusters (16, 17). The neutralization test (NT) assay is considered to be the gold standard for detecting antibodies specific for different serotypes of DENV (18). Neutralization test requires sophisticated equipment in biosafety class 3 laboratories (BSL-3), skilled technical staff and long periods of time to perform the tests (19).

To predict the severity of DENV infections, detection of serotype-specific neutralizing antibodies using neutralization test assay in various regions of the country is necessary; since the risk of severe disease is greatest during secondary, heterotypic infections in areas with more than one circulating serotype. Therefore, this study was aimed to generate data on serotype-specific antibodies against DENV in areas of northwest Ethiopia where no data were previously available.

## Method

### The study area, period, and population

A cross-sectional study was carried out among febrile patients

who were attending hospitals in Metema and Humera, north-west Ethiopia from January 2016 to March 2017. Metema hospital is located on the border with Sudan, 897 km North of Addis Ababa and 197 km from the city of Gondar. This town has a latitude and longitude of 12°58'N 36°12'E with an elevation of 685 meters above sea level. Humera Kahsay Abera hospital is located in northwest Ethiopia, 252 km from Gondar city and 974 km from Addis Ababa and located in the western zone of the Tigray region, bordered on the west by Sudan, and on the north by the Tekeze River which separates Ethiopia from Eritrea. This town has a latitude and a longitude of 14° 18'N 36° 37'E with an elevation of 602 meters above the sea level. The details of the basic characteristics of the study population and geographical locations of the two study areas have been presented in a prior published paper (20).

#### **Sample size, sampling technique, sample collection and storage**

Details of sample size determination and sampling techniques was indicated in our previously published paper (20). A venous blood sample was collected from each study participant in a vacutainer tube with no anticoagulant and centrifuged. The serum was separated and transferred into 1.5 ml volumes placed in a cryovial and stored in -20°C at the health facilities until transportation. For long storage, samples were transported in dry ice to University of Gondar Virology Laboratory and stored at -80°C till analysis. For molecular analysis and neutralization tests, stored samples were also transported in dry ice to the Institute of Virology, University of Leipzig, Germany.

#### **Data collection**

Six-hundred collected samples were initially screened for anti-dengue IgM and IgG ELISA (EUROIMMUN, Lübeck, Germany) and then positive samples were collected for further analysis (20). To identify acute dengue viral infections, the IgM positive samples were further screened for dengue infection using nonstructural protein 1 (NS1) antigen prior to RT-PCR while IgG positive samples were used to identify serotype-specific neutralizing antibodies using a neutralization test.

#### **Detection of dengue NS1 antigen**

Serum samples were tested for dengue virus NS1 antigen by ELISA (EUROIMMUN, Lübeck, Germany), with sensitivity and specificity close to 100%. The test was performed follow-

ing the manufacturer's instructions. Briefly, 100 µl of diluted (1:10 in serum diluents) patient serum samples, calibrators, positive and negative controls were transferred into each of the microplate wells pre-coated with anti-NS1 antibody and then incubated for 1 hr at 37°C. After washing, the wells were treated with enzyme conjugate (peroxidase-labeled anti-dengue virus NS1 antibody) and incubated for 30 minutes at 37°C. The wells were washed and chromogen/substrate solution was added into each of the microplate wells and then incubated in the dark at room temperature (18°C to 25°C) for 15 minutes. After addition of the stop solution into each of the microplate wells, the optical density (OD) readings were obtained with a spectrophotometer at wavelengths of 450 nm/620 nm. Results were interpreted in accordance with the manufacturer's (EUROIMMUN) recommendation that is RU/ml values  $\geq 11$  were considered positive for the presence of dengue NS1 antigen.

#### **Detection of viral RNA using RT-PCR**

The RNA was extracted from serum samples using QIAamp Viral RNA Mini Kits (Qiagen, Hilden, Germany) following the manufacturer's instructions. The RT-PCR targeting the nonstructural protein 5 (NS5) genes to detect flaviviruses, including dengue virus was used as described previously with some modifications (21). Briefly, viral RNA was reverse-transcribed into cDNA using superscript III reverse transcriptase (RT) and Flav-reverse primer. Subsequently, cDNA was amplified using *Taq* DNA polymerase. The following sequences of primers were used: Flav-forward (EMF1) 5'-TGGATGACSACKGARGAYATG-3', and Flav-reverse (VD8) 5'-GGGTCTCCTCTAACCTCTAG-3'. The PCR reaction conditions were as follows: an initial denaturation of 95 °C for 5 min, followed by 50 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 20 s and 72 °C for 50 s, followed by a final extension at 72 °C for 5 min. The final PCR amplified products were separated by gel electrophoresis on a 1.5% agarose gel containing ethidium bromide as a DNA staining dye. DNA samples were loaded on the gel and gel electrophoresis was run for 30 mins at 120 volts. After sufficient migration, the gel was visualized under ultraviolet light and a band on the agarose gel of the correct size was interpreted as a positive result by comparison with a standard molecular weight size marker (DNA Ladder).

#### **Neutralization test**

The anti-dengue serotype-specific neutralizing antibody titers

were determined by the 50% tissue culture infective dose (TCID<sub>50</sub>) assay based on neutralizing dose that protects 50% of the cell cultures (ND<sub>50</sub>), according to the method of Hsiung (22) using the Behrens and Kaerber formula. The neutralizing titer was determined by using a microassay. Briefly, each serum sample was inactivated at 56°C for 30 minutes and assayed in four separate runs, which corresponded to four different DENV serotype challenge viruses. Heat-inactivated test sera were serially diluted in two-fold steps in DMEM, starting at 1:10, and end with 1:160 titers into 96-well microtitre plates. Fifty microlitre of diluted serum was added to 100 TCID<sub>50</sub> of the challenge viruses (DENV1-4) in 50 µl. After incubation at 37 °C in a 5% CO<sub>2</sub> atmosphere for 1 h, 1x10<sup>4</sup> cells (100 µl supplemented with 10% FCS) were added to each well. Plates were further incubated at 37°C, 5% CO<sub>2</sub>, and 90% humidity for 8 to 13 days and stained with neutral red for colorimetric results. Serum samples with ND<sub>50</sub> values ≥ 10 (i.e., a 1:10 dilution of the serum) were used as a limit to consider a positive serum to any of the four DENV serotypes.

#### Dengue viruses and cells used in the neutralization test

Vero CCL81 VERO (ATCC® CCL-81™) cells were used for DENV-1 and DENV-2, and BHK-21 (ATCC® CCL-10) cells were used for DENV-3 and DENV-4. The DENV-1 (JN638337), DENV-2 (EU482701), DENV-3 (JN662391), and DENV-4 (JQ922560), representing each of the four DENV serotypes were obtained from Bernhard-Nocht-Institut Hamburg (Professor J. Schmidt-Chanasit).

#### Quality assurance mechanism

The quality of data was ensured using internal and external quality control measures. The procedures of all tests were performed strictly following the manufacturer's instructions. A questionnaire was pre-tested for clarity. Inclusion criteria and training were provided for each of the data collectors, and they were regularly supervised. Standard operating procedure (SOPs) for each test was strictly followed. Positive and negative control of ELISA, TCID<sub>50</sub>, and RT-PCR kits was tested alongside the sample.

#### Data analysis

Data were entered and analyzed using SPSS 20.0 statistical software and Microsoft Excel 2007. Descriptive statistic was used to calculate the frequency and percentage of dengue NS1 antigen and the serotype-specific antibodies. Data were summarized using frequency tables and figures.

#### Ethical clearance

This study was approved by the Ethical Committee of the University of Gondar Ref. VP/RCS/05/477/2015. After ethical approval, a letter of agreement and cooperation was written to selected health institutions. Prior to the data collection, written informed consent was obtained from each of the study participants or guardians of children.

## Result

A total of 600 serum samples were initially screened for anti-dengue IgM/IgG antibodies. Of these, 114 and 126 serum samples were positive for anti-dengue IgM and IgG antibodies, respectively (20). To identify acute dengue viral infection among seropositive serum samples, the study tested for the presence of dengue NS1 antigen prior to RT-PCR. Out of the 114 serum samples, 17 (14.9%) were positive for the dengue NS1 Ag test (Table 1). Out of 85 samples analyzed using RT-PCR, none of them were positive.

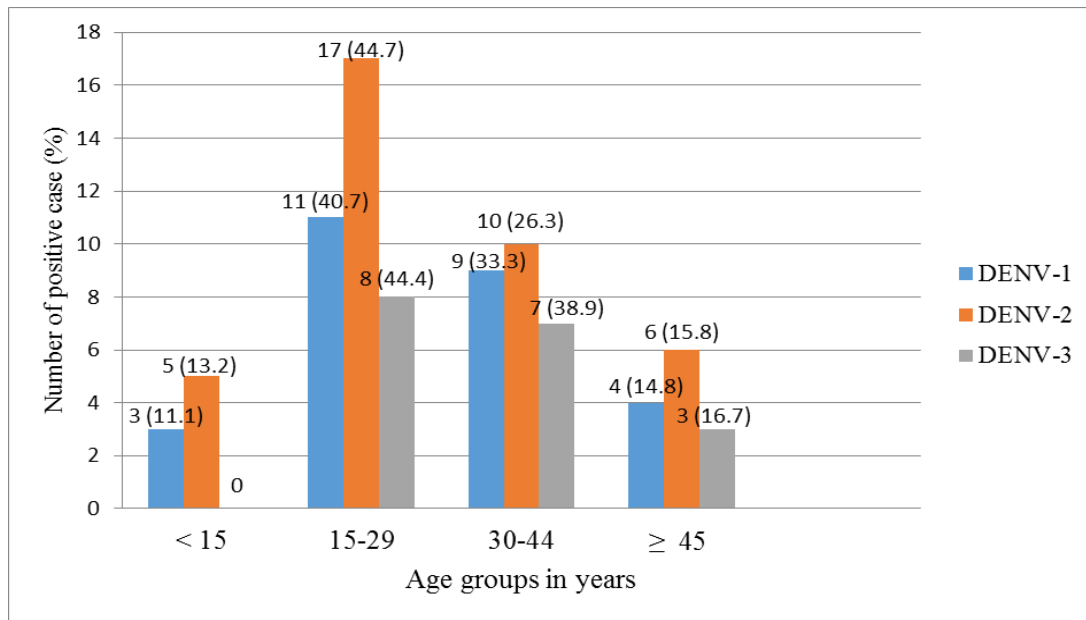
**Table 1:** Frequency of dengue virus NS1 antigen in patient samples

	Dengue NS1 antigen test	
	Total no. of samples	Positive N (%)
<b>Metema</b>	60	10 (16.7)
<b>Humera</b>	54	7 (13)
<b>Total</b>	<b>114</b>	<b>17 (14.9)</b>

Out of 126 anti-dengue IgG positive serum samples, 60 samples of sufficient volume were randomly selected and were analyzed by the neutralization test for all four DENV serotypes to determine serotype-specific neutralizing antibodies. Forty-nine (81.7%) out of the 60 dengue IgG ELISA positive samples had neutralizing antibodies for one or more DENV serotypes while seroconversion by a neutralization test was not detected in 11 (18.3%) of IgG ELISA positive samples (Table 1). Of the 49 neutralization test positive samples, 27 (55.1%) had neutralizing antibodies (NAbs) to DENV-1, 38 (77.6%) had NAbs to DENV-2, and 18 (36.7%) had NAbs to DENV-3. None of the samples had NAbs to DENV-4. In both study areas, NAbs to all the three of identified DENV serotypes were detected (Table 2). Serotype-specific neutralizing antibodies to all identified DENV serotypes were observed in all age groups except for DENV-3 in which it has not been observed in the age groups of < 15 years. In all age groups NAbs to DENV-2 was predominantly identified (Figure 1).

**Table 2:** Serum samples tested for serotype-specific DENV neutralizing antibodies.

NABs to any one of DENV serotypes						
	Total no. of samples	Positive N (%)	DENV-1 N (%)	DENV-2 N (%)	DENV-3 N (%)	DENV-4 N (%)
Metema	31	24 (77.4)	13 (54.1)	17 (70.8)	12 (50)	0 (0)
Humera	29	25 (86.2)	14 (56)	21 (84)	6 (24)	0 (0)
<b>Total</b>	<b>60</b>	<b>49 (81.7)</b>	<b>27 (55.1)</b>	<b>38 (77.6)</b>	<b>18 (36.7)</b>	<b>0 (0)</b>



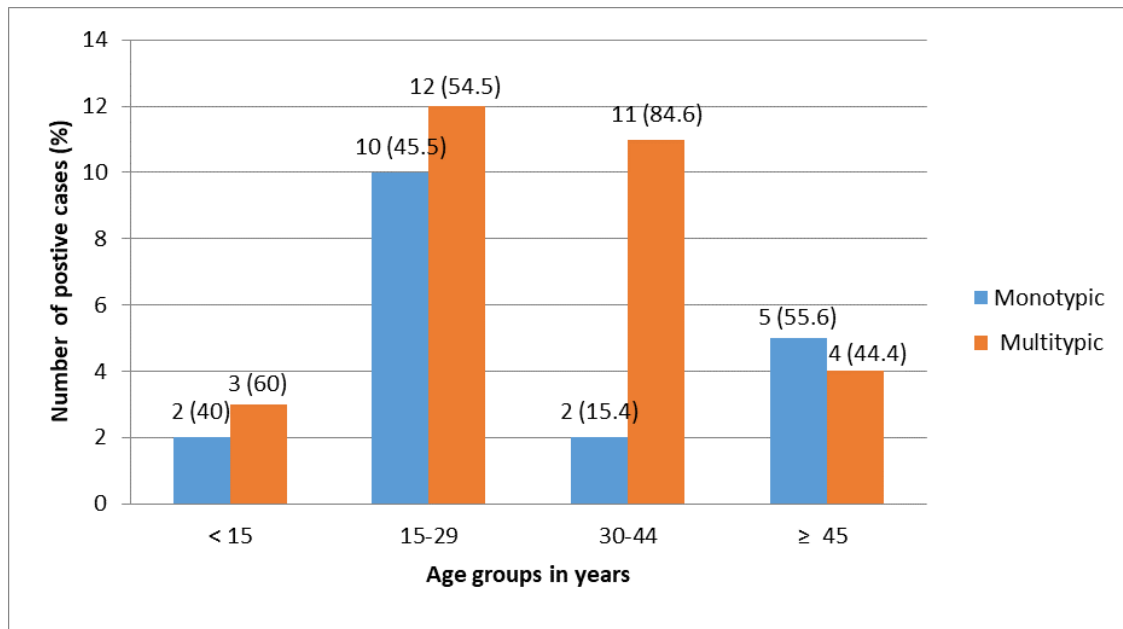
**Figure 1:** Age-wise distribution of NABs to any one of DENV serotypes.

The circulation of NABs to the three of DENV serotypes (i.e. DENV-1, -2, & -3) in the study area was confirmed by a neutralization test. The frequencies of monotypic (presence of NABs to only one of the four DENV serotypes) and multitypic (NABs to more than one serotypes simultaneously) were 19 (38.8%) and 30 (61.2%), respectively. Neutralizing antibodies to only one serotype by DENV-2 were the most frequently found 10 (20.4%), followed by NABs to more than one serotype by DENV-1 & DENV-2 16 (32.6%). Four (8.2%) out of the 49 neutralization test positive samples had NABs to all three DENV-1, -2, and -3 serotypes. Out of the 49 neutralization positive samples, 24 (49%) and 25 (51%) were found in Metema and Humera, respectively (Table 3). In all age groups, monotypic and multitypic NABs were observed (Figure 2).

**Table 3:** Frequency of monotypic and multitypic NABs to DENV serotypes.

	Metema N (%)	Humera N (%)	Total N (%)
Only DENV-1	3 (12.5)	2 (8)	5 (10.2)
Only DENV-2	5 (20.8)	5 (20)	10 (20.4)
Only DENV-3	2 (8.3)	2 (8)	4 (8.2)
DENV-1 & -2	4 (16.7)	12 (48)	16 (32.6)
DENV-1 & -3	2 (8.3)	0 (0)	2 (4.1)
DENV-2 & -3	4 (16.7)	4 (16)	8 (16.3)
DENV-1, -2, & -3	4 (16.7)	0 (0)	4 (8.2)
Monotypic	10 (41.7)	9 (36)	19 (38.8)
Multitypic	14 (58.3)	16 (64)	30 (61.2)
<b>Total</b>	<b>24 (49)</b>	<b>25 (51)</b>	<b>49 (100)</b>





**Figure 2:** Proportions of monotypic and multitypic NAbs to DENV infection

## Discussion

The study observed that in the majority of IgG ELISA positive cases, 81.7% of samples had DENV NAbs to any one of the three (1, 2, 3) DENV serotypes; this indirectly indicates the presence of DENV transmission in the study areas. Multitypic NAbs were also observed in 61.2% of the neutralization test positive samples, which suggests the presence of previous exposure to more than one DENV serotypes. These findings signify the importance of differential diagnosis of DENV infections in acutely febrile patients, especially in the area where malaria is endemic; this would help to rule out malaria and thus prevent unnecessary anti-malaria treatments. In addition to this, early diagnosis of dengue during the acute phase and identification of serotype-specific NAbs against DENV infection is also important for patient management as well as for the implementation of control measures (5).

In this study, DENV serotype-specific analysis identified NAbs to DENV-1, DENV-2, and DENV-3 providing indirect evidence that DENV-1, -2, & -3 serotypes circulate in the study areas. These results are in agreement with the circulating DENV-1, -2, & -3 serotypes reported in Kenya (23) This finding predicts the possibility of the future occurrence of the dengue complications in the study areas. As it has been well-documented, the co-circulation of different DENV serotypes is a risk factor for the occurrence of severe dengue as a consequence of sequential infection with the other serotype (24).

The study findings also observed NAbs to DENV-2 was the predominant serotype identified in all age groups in this study. This finding is consistent with the previous report, in which DENV-2 was the detected serotype during outbreaks in Dire Dawa Ethiopia, in 2013 (10). This result is also in agreement with the circulating DENV serotype, which was reported DENV-2 in Kassala State, Sudan (25). This finding likely suggests that the same serotypes may be circulating between Sudan and Ethiopia; however, it needs further confirmation by sequence analysis. The study also observed that out of IgG positives, 18.3% of samples had no NAbs against DENV. This indicates the presence of discrepancies between IgG ELISA and neutralization test data in which some samples that were positive by IgG ELISA were negative in the neutralization test. These results suggest that 11 out of 60 IgG ELISA positive samples were likely to be false-positive results. A possible reason behind this may be due to a result of the well documented serological cross-reactivity across the other flaviviruses (26).

In this study, dengue NS1 antigen was detected in 14.9% of sera, which indicates the presence of acute DENV infections among febrile patients. This is because dengue NS1 is a protein molecule present in the blood of DENV infected patients during the early clinical phase of the disease and is usually detectable in blood from the first day after the onset of fever up to 9 days (27). However, a negative NS1 antigen test results later in infection do not rule out dengue infection (28). The absence of dengue NS1 antigen among IgM positive case

is consistent with the lack of acute-phase infection; since the sensitivity of NS1 antigen detection decreases with increasing concentrations of antibody (29). Hence, it is important to detect IgM/IgG to rule in/out late dengue infection, since IgM starts 4-6 days and this persists for 2 to 3 months, and then disappears from blood, while IgG starts 10 days from onset of fever and persists for life decades (30). According to the results of this study, it was observed that many NS1 antigen-negative cases were found among anti-dengue IgM positive cases; these results are in agreement with other studies (31, 32).

Although this study detected dengue NS1 antigen in the serum samples, viral RNA using RT-PCR was detected in none of them. This may likely be due to loss of viral RNA; because of lack of facilities in Ethiopia, where samples were stored for a prolonged period. Moreover, power supplies are also limited, particularly in the study areas; this could result in repeated freeze-thawing of stored samples in refrigerators, leading to the reduced recovery of viral RNA and eventually results in false negative RT-PCR. Several studies illustrated that infections with DENV at an early stage can be detected in both NS1 antigen test and RT-PCR. However, several weeks after the onset of clinical symptoms, both methods will no longer give positive results for DENV infection. Nonetheless, even years after infection, serotype-specific IgG antibodies can be detected by the neutralization test (5, 14, 15).

#### Limitations of the study

Although an attempt has been made to detect the dengue NS1 antigen, which represents a new approach to the diagnosis of acute dengue infection and to identify dengue serotype-specific neutralizing antibodies, this study has limitations. Since the dengue NS1 antigen test was done from seropositive samples, those samples, which would be positive before IgM production may be missed and thus the prevalence of NS1 antigen does not represent the whole population. The small sample size was used to identify dengue serotype-specific neutralizing antibodies since the neutralization test is a laborious technique; this limits its use in a large-scale study. Although the detection of DENV using RT-PCR was limited, it had the benefits of analyzing samples in this study by dengue NS1 antigen and neutralization tests. In spite of limitations, the study provides the first documented data on the presence of neutralizing antibodies to three of DENV serotypes in the study areas.

## Conclusion

The study confirmed the presence of dengue NS1 antigen among febrile patients attending health institutions, and also identified serotype-specific NAb to DENV-1, DENV-2, and DENV-3, with a predominance of DENV serotype 2. Furthermore, the presence of multitypic antibodies was also observed in the study cases. These findings confirm the occurrence of active transmission and past exposure to DENV infections in the study areas and thus, highlight the importance of dengue surveillance at large in the country.

#### Conflicts of interest

All authors declare that no competing interest exists.

#### Data availability

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

#### Funding

The project was funded by the University of Gondar.

#### Author's contributions

GF: participated in the conception, design and proposed the research idea, data collection, laboratory analysis, data analysis and interpretation of the findings, drafted the manuscript and wrote up. MT and EA: participated in the conception, design and proposed the research idea, data analysis and interpretation of the findings. YW: participated in data collection, data analysis and interpretation of the findings. BT and UGL: participated in the conception, design and proposed the research idea, supervision/consultation, data analysis and interpretation of the findings. All authors reviewed and approved the final manuscript.

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