# Dengue diagnosis in an endemic area of Peru: Clinical characteristics and positive frequencies by RT-PCR and serology for NS1, IgM, and IgG

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## ARTICLE INFO

## ABSTRACT

*Background:* Huánuco is a central eastern region of Peru whose geography includes high forest and low jungle, as well as a mountain range that constitutes the inter-Andean valleys. It is considered a region endemic for dengue due to the many favorable conditions that facilitate transmission of the virus.
*Methods:* A total of 268 serum samples from patients in Huánuco, Peru with an acute febrile illness were assessed for the presence of dengue virus (DENV) via RT-PCR and NS1, IgM, and IgG ELISA during December 2015 and March 2016.
*Results:* DENV was detected in 25% of samples via RT-PCR, 19% of samples by NS1 antigen ELISA, and 10.5% of samples by IgM ELISA. DENV IgG was detected in 15.7% of samples by ELISA. The most frequent symptoms associated with fever across all groups were headache, myalgia, and arthralgia, with no significant difference between the four test methods
*Conclusions:* In this study, DENV was identified in up to 25% of the samples using the standard laboratory method. In addition, a correlation was established between the frequency of positive results and the serological tests that determine NS1, IgM, and IgG. There is an increasing need for point-of-care tests to strengthen epidemiological surveillance in Peru.

## Introduction

Dengue is a major public health concern with up to 3.9 billion people in 128 countries at risk of the disease (Moreira et al., 2018; Brady et al., 2012). It is the most rapidly spreading mosquito-borne viral disease, with a 30-fold increase in global incidence over the past five decades (World Health Organization, 2012). The World Health Organization (WHO) estimates that the incidence of dengue virus (DENV) infection is about 50 million to 100 million cases per year (WHO, 2009), with more than 100 endemic countries and a documented further spread to previously unaffected areas (Mood and Mardani, 2017). In the Americas alone, the last report in 2015 mentioned 2.35 million DENV infections, of which 10 200 cases were considered as severe dengue, with 1181 deaths (World Health

Organization, 2018). Despite these alarming figures, this is likely an underestimate of the real prevalence and burden of the disease, given the underreporting, misclassification, and limitations of the health systems in some countries, particularly those within Africa and the Americas (Suaya et al., 2009; Beatty et al., 2011). Peru is included in the group of the 20 most highly endemic countries in the world according to figures reported to the WHO until 2010 (World Health Organization, 2012). Huánuco is a region of Peru considered endemic for DENV, and in 2016, the region of Huánuco reported its highest incidence in the last decade (Centro Nacional de Epidemiología, 2018).

The transmission of DENV is maintained through a human–mosquito–human cycle that involves mosquitoes of the genus *Aedes*, principally *Aedes aegypti* (Mood and Mardani, 2017; Omokoko et al., 2014). There are four antigenically related but distinct dengue viruses, described as serotypes DENV-1, 2, 3, and 4. All four serotypes are capable of causing an infection that induces a primary immune response, leading to long-term protection against only the homologous serotype. However, if a secondary infection with a different serotype occurs, the immune response can lead to a more severe dengue fever presentation in some cases (Megawati et al., 2017). The incubation period ranges from 3 to 14 days and symptoms usually develop between 4 and 7 days after the vector bite (Fukusumi et al., 2016). In 2009, the WHO introduced a revised classification scheme comprising three categories: dengue without warning signs, dengue with warning signs, and severe dengue that includes the clinical features of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (World Health Organization, 2009). It is estimated that 50 million to 100 million cases per year of infection with DENV manifest with symptoms (Bhatt et al., 2013). Of these clinically apparent DENV infections, most affect adult patients because DENV infections are commonly asymptomatic or minimally symptomatic in children (Endy, 2002; Cobra et al., 1995).

One of the main requirements for control of the disease is timely and accurate laboratory diagnosis. The diagnosis can be made via nucleic amplification, serology, or antigen detection. Reverse transcriptase PCR (RT-PCR) is a highly sensitive and specific diagnostic tool that can be used in both clinical and public health surveillance settings, due to its ability to provide a positive result during the first 5 days of the disease (Darwish et al., 2015; Santiago et al., 2013). On the other hand, immunoglobulins IgM and IgG and non-structural protein 1 (NS1) are commonly used in serological detection. The most widely used approach is IgM ELISA because IgM can be detected as early as 4 days after the onset of the disease and in more than 80% of patients with dengue (Rai et al., 2017; Simmons et al., 2012; Teoh et al., 2015). The NS1 antigen can be detected in DENV-infected patients from day 1 up to day 18 after symptom onset and high levels of circulating NS1 correlate with disease severity (Buonora et al., 2017). NS1 can be detected either directly by lateral flow rapid test, also known as immunochromatographic assay (Ambrose et al., 2017), or by ELISA until 9 days later, when RNA detection becomes negative (Sawant et al., 2017).

This study was performed to evaluate the usefulness and applicability of diagnostic tools, i.e., RT-PCR and NS1, IgM, and IgG ELISA, in patients with clinically suspected dengue in an endemic region of central eastern Peru.

## Materials and methods

### Study location

The study was conducted in the region of Huánuco during December 2015 and March 2016 within primary health care centers managed by the Leoncio Prado Health Network of the Ministry of Health in Peru and within the Contingence Hospital of Tingo María. Patients who fulfilled the inclusion criteria were recruited. The primary health care facilities included in the study were the Health Center Luyando, José Crespo Castillo, Mariano Damaso Beraun, Daniel Alomia Robles, Hermilio Valdizan, Monzon, and Tingo María Hospital.

### Study subjects

The inclusion criteria were patients diagnosed with an acute febrile syndrome, defined as fever higher than 38 °C for ≤7 days, without an identifiable source of infection and associated with one or more of the following signs and symptoms: headache, myalgia, arthralgia, retro-ocular pain, lower back pain, cutaneous rash, hyporexia, odynophagia, nausea, emesis, abdominal pain, asthenia, syncope, hypothermia, and jaundice.

Signs suggestive of coagulopathies (epistaxis, bleeding gums, petechiae, ecchymosis, bloody sputum, hematemesis, and melena) and signs indicating central nervous system involvement (neck rigidity and altered mental state) were also considered.

Patients of both sexes were included, without any age restriction. The exclusion criteria were patients with an incomplete record of their medical data and patients with an identifiable source of infection, such as an acute upper respiratory tract infection, pneumonia, sinusitis, or urinary tract infection, among others. Physicians used a standardized questionnaire to collect demographic and clinical symptom information.

### Ethics statement

This study was approved by the Research Ethics Board of the Hospital Regional Docente de Cajamarca, Peru. Informed consent was obtained from all of the study participants according to the research protocol. For underage participants, informed consent was obtained from their parents or their respective guardians before enrollment.

### Samples

A total of 268 samples were collected using Vacuette Serum Separator Clot Activator tubes (Vacuette, Greiner Bio-One, Kremsmünster, Austria). All samples were stored at −80 °C after collection and transported to Lima (Peru) under standardized frozen conditions for further molecular assays.

### RNA extraction

RNA extraction from 200 μl of the serum sample was performed using the High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany), following the manufacturer's instructions. Viral RNA obtained after extraction was eluted in 100 μl of nuclease-free water and then processed or stored at −20 °C until use.

### Real-time RT-PCR assay for the detection of DENV and serotypes with TaqMan probe

A one-step RT-PCR was performed using TaqMan with BHQ quencher probe at 125 nM and 250 nM of primers in a final volume of 20 μl. Five microliters of the extracted RNA was combined with 15 μl of the Master Mix and the reverse transcription step was performed as follows: 95 °C for 15 min, 60 cycles of 15 s at 95 °C and 45 s at 60 °C. All procedures were performed in a LightCycler 2.0 instrument and data were analyzed with LightCycler software 4.1 (Roche Diagnostics, Mannheim, Germany). The primers and the probe used have been described previously by Leparc-Goffart (Leparc-Goffart et al., 2009) (**Supplementary Material**, Table S1).

*DENV NS1 antigen and IgM and IgG antibody ELISA*

The presence of DENV NS1 antigen was detected by Euroimmun ELISA (Euroimmun AG, Lübeck, Germany). DENV IgM and IgG antibodies were also detected using Euroimmun ELISA (Euroimmun AG, Lübeck, Germany). Each serum sample was run in duplicate, in accordance with the manufacturer's instructions.

*Statistical analysis*

The patient data were entered into a Microsoft Access database designed for this study. They were then tabulated in a Microsoft Excel file with the counting function, and the respective tables were prepared to show the data in a consolidated and summarized manner. Graphics were generated using OriginPro v.10 software. The prevalence rates of positives and negatives were analyzed using the Chi-square test. The diagnostic accuracy was analyzed to estimate confidence intervals (95% CI) for sensitivity, specificity, and two-level likelihood ratios. Age characteristics were analyzed using analysis of variance (ANOVA), adjusted to models of a normal distribution and represented in a box-chart. The matrices of clinical signs and symptoms were compared using the paired *t*-test. The correlation scatter matrices were established with the Pearson coefficient (*r*) and the area established at the 95% CI. Differences were considered statistically significant if the *p*-value was <0.05.

**Results**

In this study, a total of 268 samples from patients diagnosed with an acute febrile illness (AFI) were analyzed to determine the frequency of DENV infection.

Figure 1 shows that the frequency of DENV as determined by RT-PCR method was 25.75%; this was significantly higher ($p < 0.05$) than the frequencies obtained by ELISA for NS1 (19.03%) and IgM (10.45%). The frequency of positive IgG (15.67%), indicating a past or secondary infection by DENV, is also shown independently. Thus, the RT-PCR method was used as the reference method in the subsequent analyses (**Supplementary Material**, Table S2).

The diagnostic accuracy of the methods used was evaluated by determining the sensitivity and specificity indexes (Table 1). Comparison of the RT-PCR test with the immunological tests based on the detection of the NS1 antigen and IgM showed sensitivities of 42% and 13%, respectively. These values indicate the conditional probability of a positive diagnosis by means of the respective ELISA test with respect to the positive cases by RT-PCR for DENV. On the

other hand, the conditional probability of obtaining a negative result by immunological tests with respect to negative cases by RT-PCR corresponds to the specificity of the immunological tests; these showed values close to 90%. However, regarding the immunological tests, the diagnosis of DENV by NS1 was found to be superior because the probability of finding a positive case was about four times higher than in the group of subjects affected by an AFI without DENV infection.

Finally, from a detailed analysis of the diagnostic tests for DENV (Table 1), it can be concluded that the diagnostic accuracy ordered from highest to lowest is as follows: RT-PCR > NS1 ELISA > IgM ELISA.

In this study, no significant biases were found for the diagnosis of DENV according to the sex of the patient (Figure 2). Also, the age of the patients was not a determinant associated with DENV infection (Figure 3). Figure 3 shows that the age distribution of those affected by an AFI corresponded to a normal distribution (ANOVA, $p < 0.05$), and a similar distribution was obtained for cases with a positive diagnosis for dengue regardless of the diagnostic method used (**Supplementary Material**, Table S3).

The population affected by an AFI corresponded to an adult population with a mean ± standard deviation age of 26 ± 16 years (95% CI 24–28 years; median 22 years). The population with a positive diagnosis of DENV by RT-PCR was characterized by an average age of 28 ± 16 years (95% CI 24–32 years; median 26 years). Similarly, in the populations found positive by immunological methods via the detection of NS1 and IgM, the majority corresponded to an adult group with a mean age of 30 ± 17 years (95% CI 25–35 years; median 28 years) and 28 ± 15 years (95% CI 22–34 years; median 27 years), respectively. Other statistical parameters of the populations studied are shown in Figure 3.

An important aspect in the determination of the etiology of the disease is the clinical diagnosis based on signs and symptoms. When the frequencies of signs and symptoms were compared between the total population of AFI patients and the patients diagnosed with DENV, no significant differences were observed (Figure 4a). This is clearly shown through correlation analysis between the total AFI population and the AFI population with a diagnosis of DENV infection (Figure 4b), in which highly positive correlations were observed. However, more detailed analysis with consideration of the odds ratio allowed some differences to be established for the patients diagnosed with DENV (Figure 4c). Thus, the odds ratio of RT-PCR was found to be significantly different compared to the diagnosis by NS1 (paired *t*-test, $p = 0.036$). Although the odds ratio varied in a similar way and the correlation was acceptable (Figure 4d), a higher incidence of headache, myalgia, and odynophagia in the population diagnosed by RT-PCR could be responsible for these differences, by having a greater strength of positive association with the presence of dengue confirmed by the test. RT-PCR and NS1 showed clear differences compared to the IgM-positive cases (paired *t*-test, $p = 0.012$ and $p = 0.004$, respectively) (**Supplementary Material**, Table S4). These differences correspond to negative correlations (Figure 4d), which can be explained by the higher incidence of signs and symptoms that are infrequent in the population with AFI (e.g., petechiae, bleeding gums, bloody sputum, chest pains, etc.). These minority signs and symptoms appeared in patients diagnosed with IgG and although their incidence was lower than in patients diagnosed with IgM (paired *t*-test, $p = 0.003$), they are directly related to a secondary infection by DENV. The same clinical trend was observed in these groups of patients according to the correlation coefficient obtained for both ($r = 0.6178$). Overall, this indicates that a matrix based on signs and symptoms is too complex for a presumptive diagnosis of dengue infection.
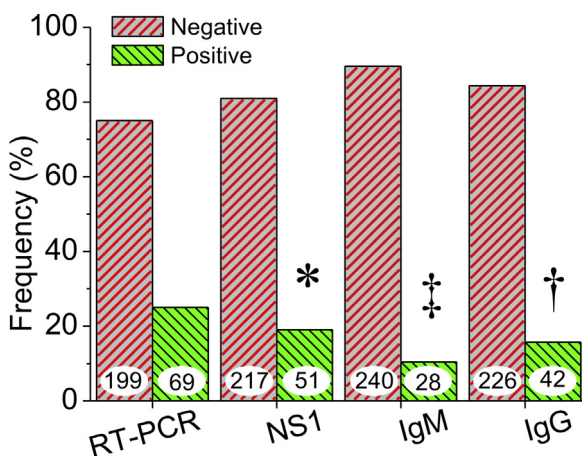


**Figure 1.** Frequency of dengue virus in the affected population with an acute febrile illness (AFI) during 2016 (Huánuco, Peru).

**Table 1**
Diagnostic accuracy; RT-PCR is the gold standard or diagnostic of reference.

| | Index | Reference test | | |
| --- | --- | --- | --- | --- |
| | | RT-PCR[a] | NS1[a] | IgM[a] |
| Diagnostic test | NS1 | | | |
| | Sensitivity | 0.420 (0.311–0.538) | | |
| | Specificity | 0.889 (0.838–0.926) | | |
| | PLR | 3.80 (2.35–6.16) | | |
| | NLR | 0.65 (0.53–0.80) | | |
| | DOR | 5.83 (3.04–11.19) | | |
| | IgM | | | |
| | Sensitivity | 0.130 (0.070–0.229) | 0.412 (0.287–0.548) | |
| | Specificity | 0.904 (0.856–0.938) | 0.968 (0.935–0.984) | |
| | PLR | 1.37 (0.65–2.88) | 12.76 (5.74–28.39) | |
| | NLR | 0.96 (0.87–1.06) | 0.61 (0.48–0.77) | |
| | DOR | 1.42 (0.61–3.31) | 21.00 (8.23–53.60) | |
| | IgG | | | |
| | Sensitivity | 0.362 (0.259–0.480) | 0.765 (0.632–0.860) | 0.679 (0.493–0.821) |
| | Specificity | 0.914 (0.868–0.946) | 0.986 (0.960–0.995) | 0.904 (0.860–0.935) |
| | PLR | 4.24 (2.44–7.37) | 54.55 (17.55–169.52) | 7.08 (4.45–11.27) |
| | NLR | 0.70 (0.58–0.84) | 0.24 (0.14–0.39) | 0.36 (0.21–0.61) |
| | DOR | 6.08 (3.02–12.23) | 228.58 (61.65–847.56) | 19.92 (8.08–49.09) |

PLR, positive likelihood ratio; NLR, negative likelihood ratio; DOR, diagnostic odds ratio.
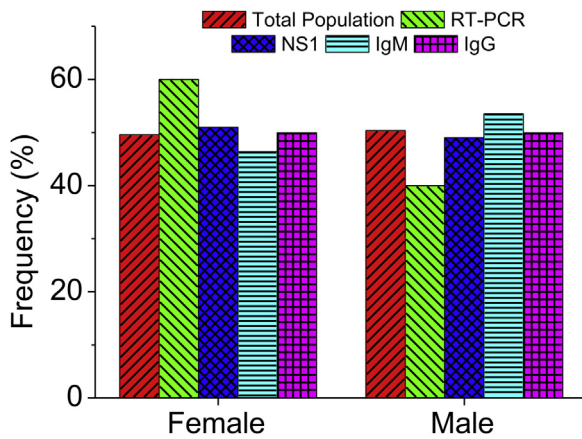[a] Values are presented with the 95% confidence interval in parenthesis.



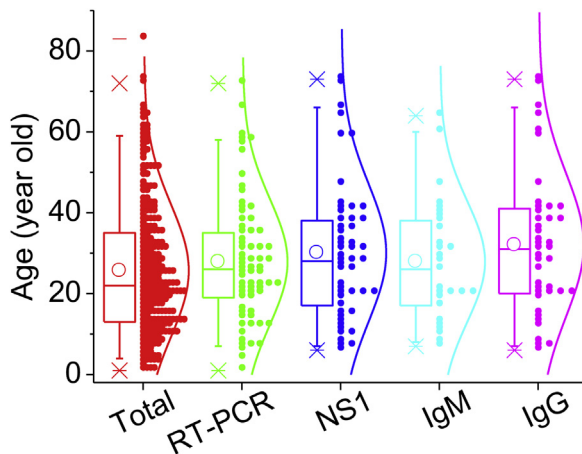**Figure 2.** Distribution of the frequency of female and male patients.



**Figure 3.** Box-chart of the age distribution.

Of the 69 samples that were positive by RT-PCR, 52 were serotype DENV-2 and 17 could not be typed, which constitutes a limitation of this study.

## Discussion

Peru is one of the 100 countries affected with dengue fever. During 2016, Peru registered a total of 31 868 cases of DENV infection, of which 3855 were dengue with warning signs and 124 were severe dengue cases, and there were 41 deaths secondary to DENV (Centro Nacional de Epidemiologia, 2018). Huánuco is a region located in central Peru that has experienced a two-fold increase in its population during the last 7 years, with 172 924 inhabitants in the last report from 2014 (INEI). Furthermore, Huánuco is one of the Peruvian localities with the highest *Aedes* index, which ranges from 1.4% to 11.69% (Centro Nacional de Epidemiologia, 2018).

In the present study, a total of 268 serum samples from patients with an AFI were assessed for the presence of DENV using molecular diagnostic methods during December 2015 and March 2016. A total of 69 (25.7%) positive DENV cases were identified, of which 46 (66.7%) occurred during January and February. This finding correlates with the peak incidence in Huánuco, described as occurring during the sixth epidemiological week by the Peruvian Ministry of Health (Centro Nacional de Epidemiologia, 2018). Furthermore, it can be explained by the increase of 2 °C in maximum local temperature (SENAMHI, 2016) and the increase in precipitation rate (accumulated rainfall of 300 mm/week) reported in Huánuco during January and February of 2016 (Centro Nacional de Estimación, 2016), which may have created better climate conditions for the transmission of DENV by the *Aedes* mosquitoes.

In relation to the diagnosis, several studies reported in the literature have assessed the usefulness of the available diagnostic methods to identify DENV. IgM ELISA is one of the most widely used techniques in many public health care centers (Hunsperger et al., 2014).

A case–control study comparing seven commercial antigen antibody ELISAs for the detection of DENV described sensitivity values ranging from 86% to 91% and specificity values ranging from 82% to 93% for Panbio IgM ELISA, and values of 81–88% and 93–99%, respectively, for Standard Diagnostics ELISA IgM, with a 95% confidence interval (Suleman et al., 2016). In an analysis by Hunsperger et al. (2014), the sensitivity of Venture E ELISA IgM was 97% for primary DENV infections compared to 96% for secondary DENV infections, and the specificity was 84% against the DENV-negative panel (Hunsperger et al., 2014). However, other studies have reported lower diagnostic accuracy values, such as a study from Malaysia by Kassim et al., in which a sensitivity of 40.9% was described for Panbio IgM ELISA (Kassim et al., 2011), and a study
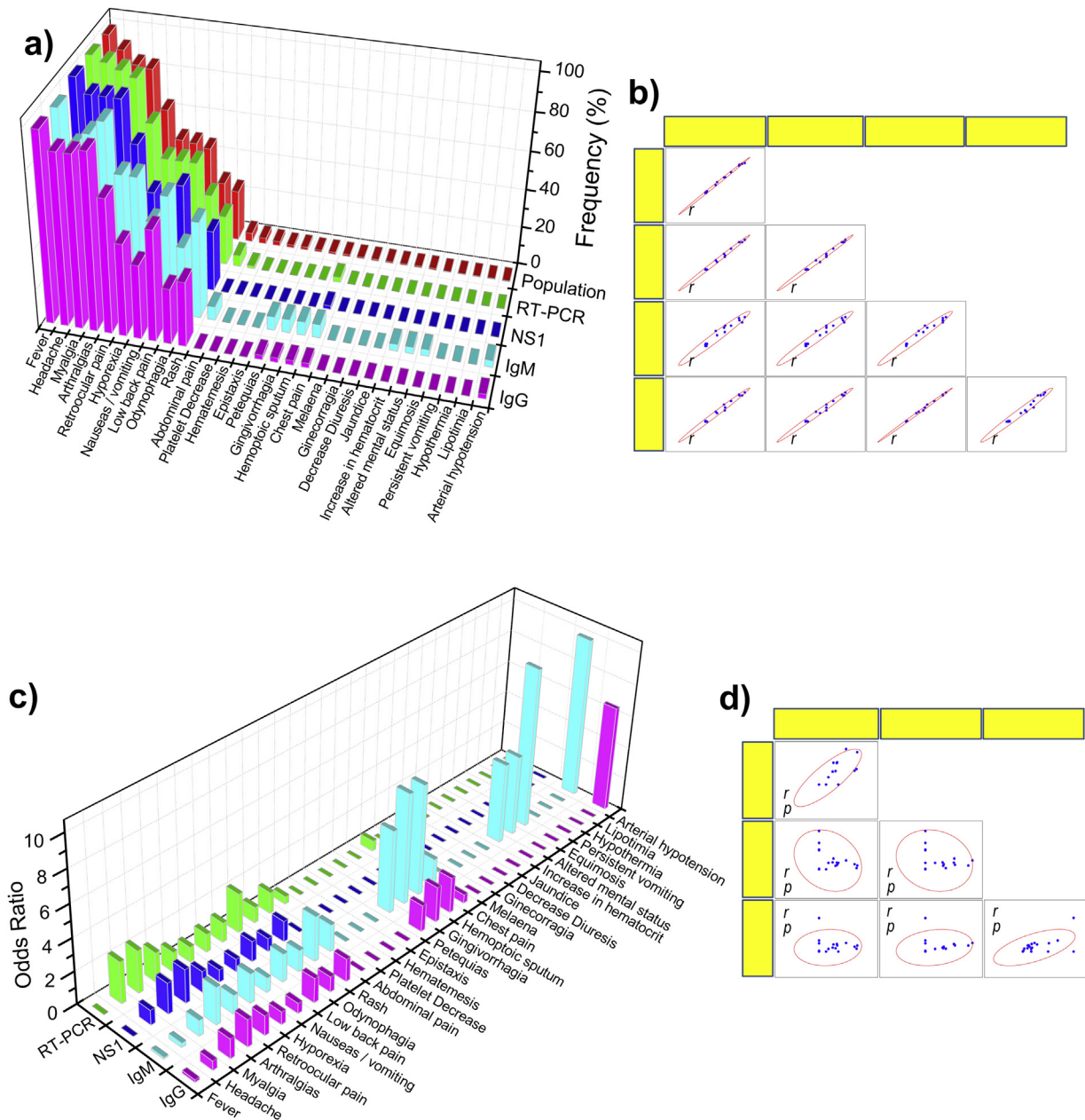
**Figure 4.** Signs and symptoms associated with a presumptive diagnosis of dengue virus infection.
Frequency distribution (a) and odds ratio (c) of signs and symptoms. Correlation analysis between frequencies (b) and odds ratio (d) of the signs and symptoms.

published in 2016 by Ashwini et al. that reported a 47.9% positive detection rate for IgM ELISA (Anand et al., 2016). In the present study, a sensitivity of 13.0% and specificity of 90.4% was found for IgM ELISA when compared with RT-PCR as the reference test. The lower sensitivity and specificity values may be explained by the differences in performance characteristics related to patient infection status. Primary DENV infections typically have a stronger and more specific IgM response; consequently, IgM-positive rates in secondary infections are not as high as in primary infections, and in some secondary DENV infections, IgM may not be detected at all, as evidenced in the medical literature (Brady et al., 2012; Chanama et al., 2004).

NS1 is a multifunctional glycoprotein that plays important roles in immune evasion, pathogenesis, and the early stage of viral replication (Scaturro et al., 2015). It can be detected in the sera of infected patients during the acute phase of illness, which typically refers to the first week of illness, although some reports indicate that it can be detectable up until 9–10 days after the first appearance of signs and symptoms (Suleman et al., 2016; Guzman et al., 2010; Krishnananthasivam et al., 2015). The sensitivity of NS1 detection depends on the technique used and whether the sample corresponds to a primary or secondary infection. In primary infection, the sensitivity can exceed 90%, while in secondary infection, the sensitivity is lower and ranges from 60% to 80% (Guzman et al., 2010; Hunsperger et al., 2016). Gaikwad et al. reported a concordance between ELISA NS1 and RT-PCR of 100% from day 3 to day 8 (Sawant et al., 2017). Ahmed et al. reported the sensitivity, specificity, positive predictive value, and negative predictive value of NS1 ELISA to be 73.53%, 100%, 100%, and 70%, respectively (Shrivastava et al., 2011), while Hunsperger et al. reported 60–75% sensitivity and 71–80% specificity for NS1 ELISA (Hunsperger et al., 2014). The present study found a sensitivity of 42.0%, specificity of 88.9%, positive likelihood ratio (PLR) of 3.8, and negative likelihood ratio (NLR) of 0.65 for NS1 ELISA; this was the

lowest NLR value of all the diagnostic methods evaluated when compared with RT-PCR. Although the reported sensitivity of NS1 ELISA was almost four-fold that of IgM ELISA, the value remains lower than those reported in the cited literature. This could be the result of immune complex formation during secondary infection, which impairs NS1 detection by ELISA (Buonora et al., 2017). Some studies have also reported lower NS1 ELISA sensitivity in DENV-4 infections compared with infections caused by other serotypes, in both primary and secondary infections (da Costa et al., 2014).

Patients with symptomatic DENV infections go through three clinical phases: a febrile phase, a critical phase, and a recovery phase. The febrile phase is characterized by sudden high-grade fever, usually $\geq 38.5\,^{\circ}$C, accompanied by unspecific symptoms such as headache, myalgia, and transient macular rash or by alarming symptoms such as persistent vomiting and abdominal pain (World Health Organization, 2009). In the present study, the most common symptoms among the total study population were headache (97.1%), myalgia (94.2%), and arthralgia (91.3%), and the same frequency distribution was found in RT-PCR-positive patients, with 92.9% presenting headache, 86.19% presenting myalgia, and 85.07% presenting arthralgia. Although the literature mentions that rash occurs in approximately half of the cases, only 18 (26.09%) DENV-infected patients in the present study developed a macular rash. The lower prevalence found could be explained by the fact that rash is more common during primary infection than secondary infection (Cobra et al., 1995) and 60.87% of the studied samples corresponded to secondary DENV infections, based on the positive results of IgG ELISA in relation to the total positives by RT-PCR. The present study showed that five (7.24%) cases corresponded to dengue fever with alarming signs. All five (100%) were primary infections, and four patients (5.97%) presented abdominal pain and one patient (1.45%) developed thrombocytopenia. No cases of severe dengue and no deaths occurred. As mentioned before, when the frequencies of signs and symptoms were compared between the total AFI patients and those who were positive for DENV, no significant differences were observed (Figure 4a). Also, the correlation scatter matrices made for clinical symptoms and diagnostic methods showed a positive correlation ($r > 0.98$) when the symptom frequencies were compared (Figure 4b) and a negative correlation between IgM ELISA and RT-PCR ($r = -0.23$) and between IgM ELISA and NS1 ELISA ($r = -0.14$) for symptom odds ratios (Figure 4d). Overall, it was indicated that the matrix based on signs and symptoms was too complex to establish any statistically significant relationship between clinical manifestations of DENV infection and the diagnostic methods used for the detection of DENV in the present study.

## Conclusions

In this study, DENV was identified in up to 25.74% of patients with an AFI via the detection of DENV RNA by RT-PCR. In addition, a correlation was established between the frequency of positive results and the serological tests that determine NS1, IgM, and IgG. It was found that there was a greater correlation between RT-PCR and NS1 ELISA for the diagnosis of acute DENV infection. There is a growing need for confirmatory diagnostic laboratory tests for clinically defined dengue cases to strengthen epidemiological surveillance in endemic areas.

Finally, it is concluded that the diagnostic accuracy of the tests investigated can be ordered from highest to lowest as follows: RT-PCR > NS1 ELISA > IgM ELISA.

Circulating serotypes of DENV should be monitored closely for the prevention of disease with alarming signs and serious illness.

## Limitations

The main limitation of this study is that the study design focused only on the detection of DENV, thus other potentially circulating pathogens such as chikungunya virus, Zika virus, Mayaro virus, and Oropouche virus, which have been described in Peru and can cause AFI, cannot be excluded. These arboviruses, although less common than DENV, might have been present in the DENV-negative samples or could have been present as co-infections. Additionally, the causality between the different serotypes and clinical presentation could not be established. Finally, it was only possible to classify 52 of the 69 positive samples, leaving 17 samples that were positive for DENV but not typed.

## Ethical approval and consent to participate

This study was approved by the Research Ethics Board of the Hospital Regional Docente de Cajamarca, Peru. Informed consent was obtained from all study participants according to the research protocol. For underage participants, informed consent was obtained from their parents or their respective guardians before enrollment.

## Conflict of interest

On behalf of all authors, the corresponding author states that there are no conflicts of interest or funding related to this study.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijid.2019.01.022.

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