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Detection of dengue virus serotype 3 in Cajamarca, Peru: Molecular diagnosis and clinical characteristics

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ABSTRACT

Objective: To describe and molecularly characterize an outbreak of dengue virus (DENV) infection in Cajamarca, an Andean region in Peru.

Methods: A total of 359 serum samples from patients with acute febrile illness were assessed for the presence of DENV *via* RT-PCR, ELISA NS1, IgM and IgG in Cajamarca, Peru from January 2017 to June 2017. The evaluation of the different diagnostic tests and their applicability was performed.

Results: Dengue virus was detected in 24.7% of samples by RT-PCR. Meanwhile, serological analysis detected 30.3% positive cases *via* ELISA NS1 antigen, 16.7% *via* ELISA IgG and 9.7% *via* ELISA IgM. Most of the cases corresponded to DENV-3 (77.5%). The use of RT-PCR performed better in primary infections ($P < 0.01$), while detection of ELISA IgM performed better in secondary infections ($P < 0.01$). The combination of NS1 and IgM performed better than the other assays in detecting primary (92.5%) and secondary infections (96.6%). The most frequent symptoms associated with fever were headaches, myalgias, and arthralgias across all groups.

Conclusions: We report an important outbreak of dengue infection caused by DENV-3 in Cajamarca, Peru. Our findings encourage the use of NS1 antigen and IgM co-detection. These findings demonstrate an increasing expansion of DENV-3 in Peru and highlight the importance of molecular diagnosis and serotype characterization among the clinically defined dengue cases to strengthen the Peruvian epidemiological surveillance.

KEYWORDS: Dengue; DENV; Arbovirus; Diagnosis; Peru

1. Introduction

Dengue virus (DENV) infection is currently the most rapidly spreading mosquito-borne viral disease worldwide[1]. There are 4 serotypes, referred to as DENV 1-4, which are genetically similar but differ antigenically[1,2]. This virus is responsible for approximately 390 million infections every year and accounts for more than 500 000 hospitalizations and 25 000 deaths around the world[2,3]. It is estimated that the Americas contribute to 14% of the global burden of DENV infections and account for approximately

Significance

Dengue virus is the most important mosquito-borne viral disease worldwide. In this study, we report the first outbreak of dengue virus serotype 3 in northern Peru. We also describe the applicability of different diagnostic tests in primary and secondary infections. These findings demonstrate an increasing expansion of dengue in Peru and highlight the importance of molecular diagnosis and serotype characterization to strengthen the Peruvian epidemiological surveillance.

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13 million clinically significant infections every year, which is the second region with the highest number of cases following Africa[3]. Peru is considered among the 30 most highly endemic countries as reported by the World Health Organization (WHO) between 2004 and 2010[4]. In 1990, the DENV-1 was introduced in Peruvian territory, since then all four serotypes have been described in Peru[5]; however, the majority of cases correspond to serotypes 2 and 4[5–7], being the serotype 3, are the less commonly reported[8].

The clinical presentation of the DENV infection can range from a mild fever to classical dengue fever with hemorrhage and/or dengue shock syndrome[1]. DENV infection causes a clinical course divided into three phases: febrile, critical and convalescence phase[1,2]. Classical dengue fever presents 4 to 10 days following the bite of an infected *Aedes aegypti* mosquito[1,2]. Most of the DENV-infected patients make a full recovery posterior to the febrile phase and do not enter the critical phase[1]. However, a minority of patients worsen at the time of defervescence, usually from day 4 of the disease[10], and these patients can deteriorate clinically and progress to severe dengue, characterized by bleeding with or without vascular leakage and organ failure. Infection provides protective immunity against the specific infecting serotype, however, secondary infection with a different serotype may increase the risk of a more severe clinical presentation[9–11].

Diagnosis of DENV infection relies on the timely detection of different biomarkers, as the clinical picture may be indistinguishable from other arboviruses such as Zika or Chikungunya and other febrile diseases such as Leptospirosis[12]. The ideal period for diagnosing a DENV infection is from the onset of fever to 10 days post-infection[13]. The laboratory detection of dengue can be performed by the identification of direct viral components such as viral RNA by RT-PCR or the identification of the non-structural protein 1 (NS1) and by serological detection of virus-specific antibodies[1,13]. Direct virus detection could potentially be used for early, definitive and serotype-specific identification of dengue infections during the acute phase of the disease; however, it is not routinely performed for surveillance[13]. Serological tests such as NS1 antigen and antibodies detection are used more frequently due to their ease of use compared to other techniques[1,2,11,13]. The applicability of each test varies according to the day of infection and immune status of the patient regarding past infections by DENV, as different patterns of antibody responses and viremia are observed when patients experience a primary or secondary infection[11,13].

This study aimed to describe and molecularly characterize an outbreak of DENV infection in Cajamarca, Peru, also, to identify the most common serotypes involved and describe the applicability of different diagnostic tests in the diagnosis of primary and secondary cases.

2. Subjects and methods

2.1. Study location

The study was performed in the region of Cajamarca in northern Peru, located at an altitude between 319 and 4 496 meters above sea level, with a mean of 2 720 meters above sea level. It is the fifth most populated region in Peru, with approximately 1 300 000 inhabitants, of which 64.6% live in rural areas and 35.4% in urban areas[14]. Particularly, this study was performed in the province of Contumaza, which is located at an altitude of 2 674 meters above sea level. According to recent estimates from the Ministry of Health, 1 339 cases suspicious for dengue have been notified in this region during the 2013–2018 period, with a peak of 420 cases in 2017[15].

2.2. Study subjects

We included patients attending to outpatient health centers from January 2017 to June 2017 with an acute febrile illness, defined as a temperature of 38 °C or higher for at least 7 days, without an identifiable source of infection and presenting at least one of the following symptoms: arthralgia, myalgia, headache, retro-ocular pain, low back pain, cutaneous rash, nausea, vomiting, diarrhea, and conjunctival injection. We also included warning signs such as abdominal pain, persistent vomiting, hepatomegaly, signs of bleeding (epistaxis, petechiae, ecchymosis, hematemesis, melena, hematuria, gynecorrhagia), signs of vascular leakage (fluid accumulation, tachycardia, weak pulse, cold extremities, low mean arterial pressure). Patients with an incomplete medical record and patients with an identifiable source of infection, such as an upper respiratory tract infection or urinary tract infection, were excluded from the study. Demographic and clinical data were recorded by the physicians using a standardized questionnaire. A flowchart summarizing the study design and all the procedures is shown in Figure 1.

2.3. Clinical samples

A total of 359 serum samples for a total volume of 2.5 mL were collected from patients using Vacuette Serum Separator Clot Activator tubes (Vacuette, Greiner Bio-One, Kremsmünster, Austria). The samples were collected between 1 to 10 days after disease onset, counted from the first day of fever onset. All samples were stored at -80 °C and transported to Lima, Peru for the detection of DENV RNA by RT-PCR, NS1 antigen ELISA, IgM and IgG antibodies.

2.4. RNA extraction

RNA extraction from 200 mL of the serum sample was performed

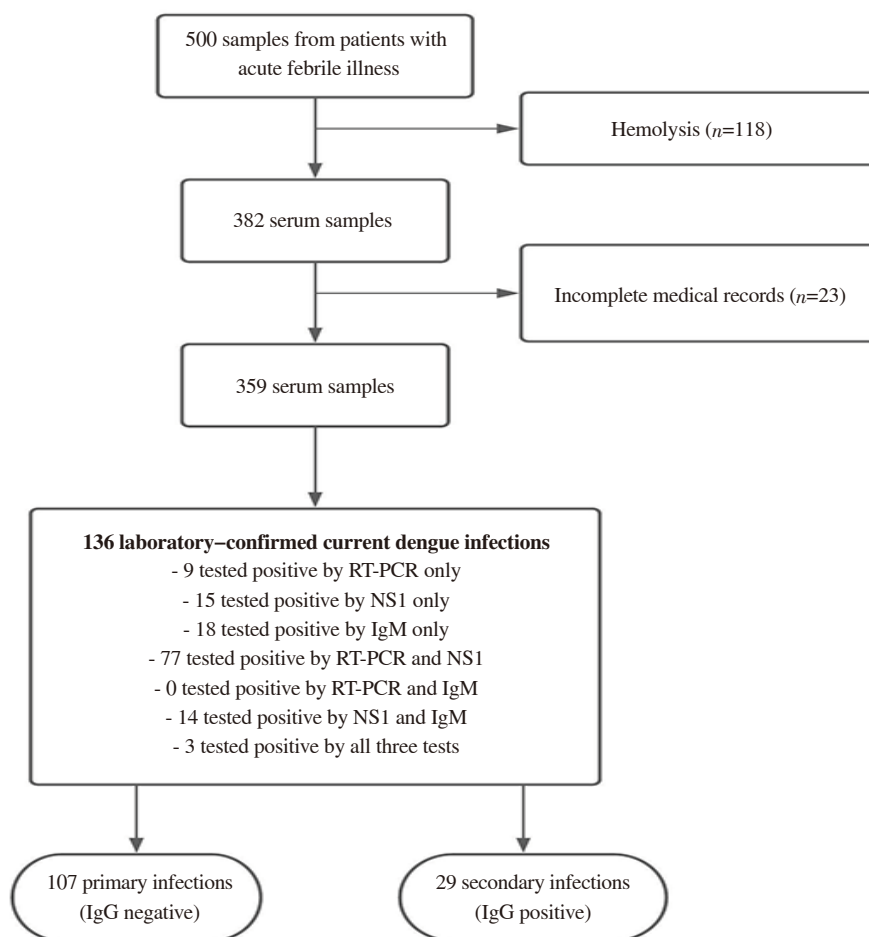


Figure 1. Flowchart of the study design and procedures.

using the High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. After extraction, the viral RNA was eluted in 100 μ L of nuclease-free water and stored at -20°C until later processed.

2.5. 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 μ L. Five microliters of the extracted RNA was combined with 15 μ L of the Master Mix and the real-time PCR 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 Leparac-Goffart[16].

2.6. DENV NS1 antigen, 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.

2.7. Definitions according to diagnostic test results

The results for the RT-PCR, NS1 antigen ELISA and antibodies ELISA were analyzed and classified according to the diagnostic interpretation proposed by Teoh *et al*[17]. Current dengue infection was diagnosed based on the detection of NS1 antigen ELISA and/or RT-PCR and/or IgM ELISA. Primary infection (first infection) was diagnosed by the detection of NS1 antigen ELISA and/or RT-PCR and/or IgM ELISA in the absence of IgG ELISA. Secondary infection (subsequent infection) was determined by the presence of IgG ELISA and a positive NS1 antigen ELISA and/or RT-PCR and/or IgM ELISA. Also, the co-detection of IgM ELISA and IgG ELISA within the 10 days of disease was considered secondary infections. Past dengue infection was determined by the detection of IgG ELISA as the only positive biomarker.

2.8. Statistical analysis

The data recollected were entered into a database using the Microsoft Excel software. All analyses and figures were carried out

using the GraphPad 9.1.1 software (San Diego, CA, USA). For the descriptive analysis of the categorical variables, the frequencies and percentages were calculated. Categorical variables were analyzed using the Pearson *Chi*-square or Fisher's exact test. The level of statistical significance was set at $P<0.05$.

2.9. Ethics statement

This study was approved by the Research Ethics Board of the Hospital Regional de Cajamarca, Peru. The samples were collected within the framework of the epidemiological surveillance program of acute febrile syndrome in the Cajamarca Region. According to international ethical guidelines for research related to human health prepared by CIOMS and WHO, ethics or informed consent is not required.

3. Results

In this study, 359 serum samples from patients with acute febrile illness were tested for NS1 ELISA, RT-PCR, IgM ELISA and IgG ELISA. Dengue virus was detected in 24.7% (89/359) of samples by RT-PCR. While, serological analysis detected 30.3% (109/359) positive cases via ELISA NS1 antigen, 16.7% (60/359) via ELISA IgG and 9.7% (35/359) via ELISA IgM (Table 1).

Table 1. Demographic characteristics according to assay [*n* (%)].

Variables	Total population <i>n</i> =359	RT-PCR (+) <i>n</i> =89	NS1 (+) <i>n</i> =109	IgM (+) <i>n</i> =35	IgG (+) <i>n</i> =60
Age (years)					
< 5	13 (3.6)	1 (1.1)	1 (0.9)	0 (0.0)	3 (5.0)
5-11	50 (13.9)	6 (6.7)	6 (5.5)	5 (14.3)	10 (16.7)
12-17	34 (9.5)	7 (7.9)	10 (9.1)	6 (17.1)	2 (3.3)
18-39	121 (33.7)	34 (38.2)	40 (36.7)	7 (20.0)	21 (35.0)
40-59	95 (26.5)	28 (31.5)	36 (33.0)	12 (34.3)	19 (31.7)
≥ 60	46 (12.8)	13 (14.6)	16 (14.7)	5 (14.3)	5 (8.3)
Gender					
Male	167 (46.5)	44 (49.4)	56 (51.4)	18 (51.4)	31 (51.7)
Female	192 (53.5)	45 (50.6)	53 (48.6)	17 (48.6)	29 (48.3)

Figure 2 shows the positive cases according to individual and combined assays. Current dengue infection was diagnosed in 37.9% (136/359) of the patients, being 107 primary infections (IgG-) and 29 secondary infections (IgG+). Most of the current dengue infections diagnosed were positive for the combination of RT-PCR and NS1 (56.6%, 77/136). Table 2 shows the diagnostic performance of each assay according to the patients' immune status regarding past dengue infections. Differences were observed in the diagnostic performance

of IgM ELISA (17.8% vs. 55.2%, $P<0.01$) and RT-PCR (74.8% and 31.0%, $P<0.01$) in primary and secondary infections respectively. NS1 antigen ELISA and the combination of the diagnostic methods showed a similar performance in both scenarios.

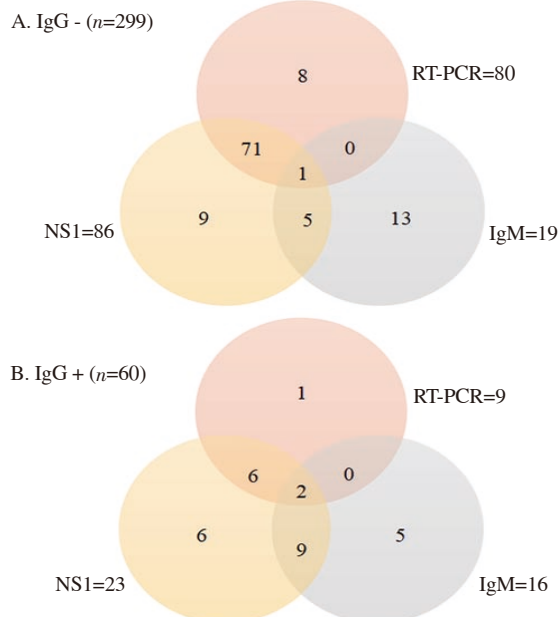


Figure 2. Diagnosis of current dengue infections according to IgG status.

The serotypes identified in the RT-PCR positive samples for dengue, DENV-2 serotype was isolated in 11.24% (10/89) and DENV-3 serotype in 77.53% (69/89) of the cases. We could not identify the serotype in 11.24% (10/89) of DENV cases.

Table 3 shows the performance of each assay according to the patient immune status and day of illness onset. It was observed that most of the cases attended within the first 4 days of disease. It was observed that RT-PCR, detected less cases in secondary infections and as patients presented with more days of disease. On the other hand, IgM detection performed better in secondary infections (3 days and above) and with more days of disease.

Clinical characteristics according to patients' immune status and diagnostic tests were described, as shown in Table 4. Among all groups, clinical symptoms were similar and only nausea was more frequent in the secondary infection group compared to the primary infection group (38.0% vs. 20.6%, $P=0.02$); however, only one case of severe dengue was identified among the secondary infections, characterized by fluid accumulation and consciousness alteration. No cases of hemorrhagic complications were reported.

Table 2. Comparison of the performance of RT-PCR, NS1 and IgM ELISA assays against dengue samples in primary and secondary DENV infection [*n* (%)].

Assay	Total <i>n</i> =136	Primary infection (IgG -) <i>n</i> =107	Secondary infection (IgG +) <i>n</i> =29	<i>Chi</i> square test	<i>P</i> -value*
RT-PCR	89 (65.4)	80 (74.8)	9 (31.0)	19.29	<0.01
NS1 ELISA	109 (80.1)	86 (80.4)	23 (79.3)	0.02	0.90
IgM ELISA	35 (25.7)	19 (17.8)	16 (55.2)	16.71	<0.01
RT-PCR/NS1 ELISA	115 (84.6)	91 (85.0)	24 (82.8)	0.09	0.76
RT-PCR/IgM ELISA	121 (88.9)	98 (91.6)	23 (79.3)	3.50	0.06
NS1 ELISA/IgM ELISA	127 (93.4)	99 (92.5)	28 (96.6)	0.59	0.44

*The test sensitivities between primary and secondary infection were compared using *Chi*-square test. Significant differences ($P<0.01$) of test sensitivities between primary and secondary infection were obtained for all tests.

Table 3. Performance of each assay for the diagnosis of current dengue infection according to day of infection and immune status [n (%)].

Days of illness	Total n=136	Primary infection			Secondary infection				
		Total n=107	RT-PCR n=80	NS1 n=86	IgM n=19	Total n=29	RT-PCR n=9	NS1 n=23	IgM n=16
1	62 (45.6)	56 (52.3)	47 (58.8)	8 (55.8)	5 (26.3)	6 (20.7)	4 (44.4)	4 (17.4)	2 (12.5)
2	29 (21.3)	22 (20.6)	19 (23.8)	19 (22.1)	3 (15.8)	7 (24.1)	3 (33.3)	7 (30.4)	2 (12.5)
3	21 (15.4)	16 (15.0)	10 (12.5)	14 (16.3)	4 (21.1)	5 (17.2)	1 (11.1)	2 (8.7)	3 (18.8)
4	9 (6.6)	6 (5.6)	2 (2.5)	2 (2.3)	3 (15.8)	3 (10.3)	0 (0.0)	2 (8.7)	2 (12.5)
5	2 (1.5)	1 (0.9)	0 (0.0)	1 (1.2)	1 (5.3)	1 (3.4)	0 (0.0)	1 (4.3)	1 (6.3)
6	4 (2.9)	3 (2.8)	1 (1.3)	1 (1.2)	1 (5.3)	1 (3.4)	0 (0.0)	1 (4.3)	1 (6.3)
7	2 (1.5)	1 (0.9)	0 (0.0)	0 (0.0)	1 (5.3)	1 (3.4)	0 (0.0)	1 (4.3)	1 (6.3)
8	5 (3.7)	2 (1.9)	1 (1.3)	1 (1.2)	1 (5.3)	3 (10.3)	1 (11.1)	3 (13.0)	2 (12.5)
9	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.4)	0 (0.0)	1 (4.3)	1 (6.3)
10	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.4)	0 (0.0)	1 (4.3)	1 (6.3)

Table 4. Clinical symptoms and warning signs in patients with current dengue infection [n (%)].

Clinical symptoms	Primary infections n=107	Secondary infections n=29	Chi square test	P-value
Headache	97 (90.7)	23 (79.3)	2.82	0.09
Myalgia	77 (72.0)	16 (55.2)	2.97	0.08
Arthralgias	73 (68.2)	16 (55.2)	1.72	0.19
Retroocular pain	54 (50.5)	14 (48.3)	0.04	0.71
Low back pain	28 (26.2)	9 (31.0)	0.27	0.60
Nausea	22 (20.6)	11 (38.0)	3.74	0.02
Rash	20 (18.7)	8 (27.6)	1.10	0.29
Vomiting	15 (14.0)	7 (24.1)	1.72	0.19
Diarrhea	11 (10.3)	2 (6.9)	0.30	0.55
Polyarthralgia	9 (8.4)	5 (17.2)	-	0.16 ^{&}
Conjunctival hyperemia	6 (5.6)	2 (6.9)	-	0.80 ^{&}
Warning signs				
Persistent abdominal pain	17 (15.9)	4 (13.8)	-	0.78 ^{&}
Hepatomegaly	1 (0.9)	1 (3.4)	-	0.31 ^{&}
Persistent vomiting	0 (0.0)	1 (3.4)	-	0.21 ^{&}
Fluid leakage	0 (0.0)	1 (3.4)	-	0.21 ^{&}
Consciousness alteration	0 (0.0)	1 (3.4)	-	0.21 ^{&}

Chi square test was performed to compare total primary vs. total secondary infections. [&]Fisher's exact test.

4. Discussion

The dengue virus and its vector, the *Aedes aegypti* mosquito, were introduced in Peru during the decade of 1990, around those years DENV-1 was first reported in the city of Iquitos[18]. Since then, different genotypes have been circulating through the country, DENV-2 was identified in 1995[19–21], DENV-3 in 2001[8] and DENV-4 in 2008[22]. The largest outbreaks in this country were produced by DENV-2 in the northern coast during the year 2000[23] and in the amazon during the year 2010[21]. According to the PAHO, the most common circulating serotypes in Peru during the last years have been DENV-2 and DENV-4[6].

In the present study, a diagnosis of current dengue infection was performed in 37.9% (136/359) of the patients according to the definitions in the methodology section. The most frequent serotype identified during this outbreak was DENV-3 with 77.5% cases among the positive cases for RT-PCR, which is the largest outbreak of this serotype reported in the region and one of the few reported in Peru. Moreover, 78.7% (107/136) of the cases were primary infections, suggesting that this outbreak occurred in a population that has not been previously exposed to dengue infection. The introduction of a new serotype could have caused this outbreak in dengue naïve subjects.

The performance of different molecular methods for the diagnosis of DENV infection, such as RT-PCR, NS1 antigen detection and

IgM detection were evaluated. The percentage of positive samples according to each method and their combination were calculated in patients with primary and secondary infections. Previous studies have reported that the sensitivity of the three tests varies in primary and secondary infections. For example, Teoh *et al.*[17] reported differences in primary vs. secondary infections: qRT-PCR had a sensitivity of 77.3% vs. 53.8%, NS1 ELISA had a sensitivity of 95.9% vs. 43.7% and while IgM ELISA 41.2% vs. 90.5%. In our study, the diagnostic performance of RT-PCR was found to be better for the detection of primary infection (74.8%) than secondary infection (31.0%), which is comparable to the findings reported in previous studies[17]. This could be explained by an earlier and faster clearance of the viremia in subjects that have been exposed to previous dengue infection and have pre-existing circulating antibodies, decreasing the time window in which viremia is present at detectable levels[11,17,24,25]. Patients with secondary infections have also been reported to have an earlier peak and faster clearance of NS1 antigenemia[24,25], however, in the present study, we found that the diagnostic performance of NS1 antigen detection was similar in primary and secondary cases (80.4% and 79.3%, respectively), which may be explained by the fact that NS1 antigenemia could possibly persist after viremia clearance[26] and even accumulate in tissues such as liver or lungs[27]. The utility of the NS1 assay has been reported previously. A study performed by Ambrose *et al.*[28] evaluated the performance of NS1 antigen assay compared to qRT-

PCR. The NS1 assay was found to have a sensitivity of 64.3%, specificity of 100%, positive predictive value of 100% and negative predictive value of 58.3%. Moreover, the application of NS1 assays may identify dengue cases which have been tested negative for IgM. A previous study reported that retrospective use of NS1 was positive for 183 of 813 that tested negative for IgM ELISA[29].

We found a better diagnostic performance of IgM ELISA for the detection of secondary dengue (55.2%) compared to primary dengue (17.8%). This finding is supported by previous evidence that suggests that the early appearance of IgM antibodies in secondary infection could lead to an improved sensitivity of IgM ELISA[1,11,17]. Also, most of the samples in the present were collected during the first days of disease onset, in which antibodies may not be circulating yet, particularly in primary cases. The best performance was reported with the combination of IgM and NS1 co-detection, with 91.9% and 96.6% positive samples in primary and secondary infections respectively. A previous study by Hunsperger *et al*[30], also showed that the combination of these diagnostic tests achieved the maximum diagnostic accuracy when performed within the first 10 days of disease in a single serum specimen. This combination could be a potential tool for most surveillance programs as it may be useful in primary and secondary infections, providing an extended window of detection in both cases. Also, these tests are affordable, require limited equipment and are easy to perform[31,32].

The performance of each assay was also evaluated regarding the day of illness onset and past dengue infections, as these factors strongly influence the dynamics of the diagnostic biomarkers. Initially, a rise of DENV viremia occurs for 24-48 hours prior to fever onset and persists for approximately 5 days of the febrile phase in primary cases; however, it is cleared earlier and faster in secondary cases[1,11,31]. On the other hand, IgM antibodies become detectable 3 to 5 days after the onset of fever and peak 6 to 10 days in primary cases but can appear as early as day 1 after fever onset in secondary cases[29]. In the present study, most of the cases were detected within the first 4 days of illness onset. When evaluating the performance of RT-PCR, we observed a poor detection of secondary cases when patients presented with more days of disease, compared to primary cases. For example, in patients that presented with 3 or more days of disease, this assay detected fewer cases among the secondary infections compared to primary infections. This could be explained by an earlier and faster clearance of viremia in secondary cases, which could occur during the first 4 days in patients with pre-existing antibodies and a memory immune response[11,24,25].

Regarding IgM detection, a higher percentage of positive samples was observed with more days of disease in both cases; however, a better performance was observed in secondary infections compared to primary infections throughout the days. This may be due to the circulation of pre-existing antibodies and the earlier production of antibodies in patients with past infections[1,13]. However, IgM titers tend to be lower during secondary infections and some cases may go underdiagnosed[13]. Contrary to RT-PCR detection, NS1 antigen detection showed a better performance in both scenarios even if patients were negative for RT-PCR. Although the kinetics of NS1 levels during secondary infections is shorter than for primary infections[24], previous studies have shown that NS1 antigen can still be positive in RT-PCR negative cases[32,33].

The clinical diagnosis of dengue can be challenging as the symptoms depend on the stage of the disease[1]. The clinical presentation can range from asymptomatic infection to severe

dengue fever with shock and/or hemorrhage. In the first few days of dengue illness, most patients present with acute febrile illness with non-specific signs and symptoms, undifferentiated from a flu-like disease: headache, myalgias, nausea/vomiting, abdominal pain and sometimes rash. Retro-orbital pain, myalgia and arthralgia are found commonly in dengue fever patients[9], the alternative name for dengue, breakbone fever, comes from the associated muscle and joint pains[34]. In our current study, the most commonly reported symptoms were headache, myalgias, arthralgias and retro-orbital pain, as reported in the literature. No differences were observed regarding symptomatology between the different diagnostic tests and primary and secondary infections.

In conclusion, we report an important outbreak of dengue infection caused by DENV-3 in Cajamarca, Peru. To the best of our knowledge, this is the first outbreak of this serotype reported in this region and one of the few reported in the country. Our findings encourage the use of NS1 antigen and IgM co-detection, as they may increase the window for detection of dengue infections regarding the day of disease and patient's immune status. These findings demonstrate an increasing expansion of DENV-3 in Peru and highlight the importance of molecular diagnosis and serotype characterization among the clinically defined dengue cases to strengthen the Peruvian epidemiological surveillance and decrease under-reporting rates in the Americas.

Conflict of interest statement

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

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Authors' contributions

MAAL, HCN, WSC, YTC, RAO, ACT and JdVM performed the PCR for virus and pathogens; WSC, LJdV and JdVM designed the study protocol. MAAL, SK and JdVM were responsible for obtaining funding and laboratory work supervision. SK, EV, LJdV, JBM, VZG, DCP and JdVM were responsible for the clinical assessment, samples collection, database completion and database analysis. HC, EV, SK, LJdV and JdVM drafted the manuscript. All authors critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

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