DEVELOPMENT AND VALIDATION OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION FOR THE DETECTION OF THE ZIKA VIRUS

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ABSTRACT

A Reverse Transcription Loop-mediated Isothermal Amplification (RT-LAMP) method was developed to detect Zika. The primers were designed based on the NS5 region of 64 complete genomes. Lyophilized LAMP reagent was used. Initially, seven different arboviruses were tested and only Zika samples tested positive. Additionally, serial dilutions of one of Zika's RNA were compared using RT-LAMP and qRT-PCR, demonstrating that RT-LAMP is 1,000 times more sensitive. We also evaluated 300 serum samples with RT-LAMP comparing the results with standard qRT-PCR methods, and we obtained a 99.3% sensitivity, 100% specificity, 100% positive predictive value, and 99.3% negative predictive value. In conclusion, this method provides a low-cost, high-performance, viable, and reliable alternative for the rapid diagnosis of Zika in primary health-care facilities.

Keywords: Zika virus; Diagnosis; RNA-directed DNA polymerase; Polymerase chain reaction (source: MeSH NLM).

INTRODUCTION

Zika is a febrile illness caused by a flavivirus called the Zika virus (ZIKV)[1]. The ability of this virus to adapt to new vectors[2] and the influence of other factors such as climate change and migration can have a major impact on its geographic expansion. The emergence, resurgence, and rapid spread of this virus is a global public health problem because of the clinical consequences that this infection can generate in vulnerable groups. Since 2015, Latin America has been the focus of attention due to the high frequency of ZIKV infections due to its tropical condition [3].

Efforts have been made to prevent, detect, and respond to the infection, and several methods have been developed for the detection of ZIKV. These methods can be grouped into enzyme-linked immunosorbent assays[4] such as ELISA, virus isolation,[5] and molecular methods such as reverse transcriptase polymerase chain reaction (RT-PCR)[6]. However, each one of thesemethods has disadvantages, such as low specificity/sensitivity (ELISA), high complexity and time (viral isolation), as well as high costs (RT-PCR)[7]. In addition, all of these methods require special laboratory conditions, making them difficult to use in most regions where ZIKV infection is prevalent. It is, therefore, necessary to find a better alternative, including more accessible diagnostic platforms and technologies that can quickly, accurately, and easily identify emerging infectious diseases at the point of care, as well as contribute to detection and surveillance.

Loop-mediated isothermal amplification (LAMP) allows one-step detection of gene amplification at a single temperature[8] and LAMP has been reported to be simpler and more sensitive than traditional PCR methods. Recently, the LAMP method has been used in a retro transcriptase format (RT-LAMP) to detect RNA virus in a single step[9]. In addition, the freeze-dried LAMP reagent does not require special conditions during storage, transportation, and operation so it can be used to detect pathogenic microorganisms even in tropical countries[10].

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If the lyophilized LAMP reagent is applicable to the detection of ZIKV, it would be very useful in various parts of the world.

In this study, a specific, sensitive, and easy-to-use method for detecting ZIKV based on RT-LAMP was developed and validated using serum samples. This method can be performed anywhere in the world requiring minimal laboratory conditions.

KEY MESSAGES

Research motivation. The introduction of the Zika virus in Peru highlights the need for diagnostic tests at the point of care, or near the patient's location.

Main findings. A low-cost, high-performance, viable and reliable diagnostic method was developed for the quick diagnosis of Zika.

Implications. The proposed diagnostic method could be a useful alternative in primary healthcare facilities in Peru.

THE STUDY

SERUM SAMPLES

Serum samples from various regions of Peru, collected under the National Surveillance System of the National Reference Laboratory for Viral Metaxenic Diseases (LRNMV) of the National Health Institute of Peru (INS), were used. These samples corresponded to patients with acute febrile clinical features. The treatment of the patients and the samples were given according to the provisions contained in the Research Protocol approved by the Institutional Committee on Research Ethics of the INS.

VIRUS IDENTIFICATION

LRNMV detected ZIKV and other arboviruses using the gold standard reverse transcriptase polymerase chain reaction (qRT-PCR) method, previously described for Zika[11], Dengue[12], Chikungunya[13], yellow fever[14], Oropouche[15] and Mayaro[16] and adapted to INS protocols.

DESIGN OF PRIMERS

The new ZIKV RT-LAMP primers were designed based on the coding gene region of the RNA polymerase dependent RNA enzyme called NS5 (non-structural protein 5) from the ZIKV genome using Primer Explorer V4 software (Fujitsu System Solutions Ltd., Tokyo, Japan). Nucleotide sequences preserved within the NS5 region were identified using a multiple alignment of 64 complete ZIKV genomes available from the DDBJ / EMBL / GenBank databases (Table 1).

EXTRACTION OF ARN

RNA extraction and purification were performed using the commercial DNA/RNA genomic purification kit GeneJET (Thermo Scientific, USA) according to the manufacturer's instructions. The optional or protocol recommended steps to eliminate the reaction inhibitors were followed.

RNA was immediately used in the RT-LAMP reaction or was stored in aliquots at -80 °C until use.

RT-LAMP TEST USING LYOPHILIZED REAGENT

Microtubes containing the lyophilized reagent were taken from a commercially available kit that targets nucleic acid by the LAMP method, the Loopamp D RNA/DNA amplification reagent (Eiken Chemical Co., Ltd., Tokyo, Japan). The microtubes were transported, stored, and rehydrated at room temperature. The RT-LAMP reaction took place in a 0.2 ml tube, with a total reaction volume of 30 µL, 5 µL of RNA solution, 1.3 µL of ZIKV RT-LAMP oligonucleotides containing FIP and BIP oligonucleotides (40 pmol), F and B (20 pmol), external oligonucleotides F3 and B3 (5 pmol), and 23.7 µL of distilled water. The cap was firmly secured and the microtube was inverted for 3 minutes to rehydrate the reagent found in the tube's cap. The tube was heated to 65 °C for 1 h and 80 °C for 5 min (enzymatic inactivation). The results of the reaction were determined visually by the color change from brown to green in the reaction solution. The control tube was considered to be negative since no evidence of a change in color was observed.

Table 1. New primers for the detection of the Zika virus by the loop mediated isothermal amplification (ZIKV RT-LAMP)

Primer	Sequence 5' to 3'
ZikV_F3	GAGATGTACTGGGTCTCTGG
ZikV_B3	GCTTTCTCAGCGCGGAT
ZikV_FIP	TTCACTGGCCTCCTAGGCCCACCATAAAAAGTGTGTCCACC
ZikV_BIP	ATGTGAATCTCGGCTCTGGCAGCGGTTACCAATGATCTTCATG
ZikV_LF	GCCCCAAGAGGAGCTG
ZikV_LB	CGCGGGCTGTGGTAAGCTG

PRELIMINARY TEST

Seven serum samples previously confirmed as positive were compared for the following viral agents: ZIKV-1 (n=2), DENV-1 (n=1), DENV-2 (n=1), DENV-3 (n=1), DENV-4 (n=1) and CHIKV (n=1) using the qRT-PCR methodologies described for the above viruses. All samples were then tested using the ZIKV RT-LAMP to assess specificity. In addition, one of ZIKV's RNA solutions was quantified and diluted in series (10 times) to 15 tubes. These dilutions were then tested by ZIKV RT-LAMP and qRT-PCR to assess sensitivity.

DIAGNOSTIC VALIDATION EXPERIMENT

A total of 300 serum samples were used to evaluate the performance of the ZIKV RT-LAMP. Half of them were positive samples of Zika. The analyzed serum samples were collected during 2016-2017 in the regions of Piura, Tumbes, Loreto, San Martín, Huanuco, Ucayali, Lima, and Cajamarca. Samples were first reassessed using the gold standard qRT-PCR method. Finally, the serum samples were tested using the ZIKV RT-LAMP method as described above and the results were compared.

STATISTICAL ANALYSIS

In order to determine the performance of the new ZIKV RT-LAMP method, diagnostic sensitivity, specificity, positive predictive value, and negative predictive value were evaluated.

RESULTS

INITIAL EVALUATION OF ZIK RT-LAMP

The new ZIKV RT-LAMP primers were evaluated with the 7 serum samples described above. The results were evident to the naked eye (Figure 1A), noting that only the positive results of the sample for ZIKV using the gold standard qRT-PCR methods were positive with ZIKV RT-LAMP. In addition, a ZIKV RNA solution was quantified obtaining an initial value of 7 ng, then diluted in series and analyzed. The ZIKV RT-LAMP test result was also determined visually (Figure 1B).

Comparative results showed that ZIKV RT-LAMP is 1,000 times more sensitive than real-time RT-PCR, revealing a detection limit as low as 0.0007 pg (Table 2).

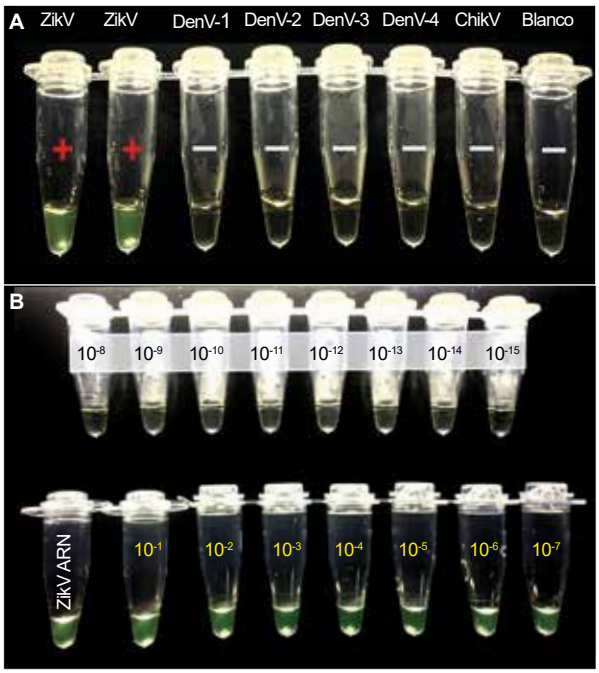

Figure 1. A. Specificity of the method for the detection of Zika virus by loop mediated isothermal amplification (ZIKV RT-LAMP), RNA solutions of ZIKV, DENV-1, DENV-2, DENV-3, DENV-4 and CHIKV LAMP positive (+) and negative (+) reaction judged visually. B. Sensitivity of the ZIKV RT-LAMP method, ZIKV RNA solution followed by a series of dilutions 10 times

ZIKV RT-LAMP LABORATORY VALIDATION

A total of 300 serum samples were analyzed using the gold standard qRT-PCR methods and 150 were determined to be positive for ZIKV, 15 for DENV-1, 15 for DENV-2, 15 for DENV-3, 15 for DENV-4, 20 for CHIKV, 10 for yellow fever, 10 for Oropouche, 10 for Mayaro, and 40 were negative for the previous ones. Afterwards, all samples were analyzed with the ZIKV RT-LAMP method. The results using both methods coincided perfectly, except for one sample that was reported as positive for ZIKV by the gold standard qRT-PCR method and negative by the ZIKV RT-LAMP method (Table 3). Finally, the competence of the ZIKV RT-LAMP method was calculated with a sensitivity of 99.3% (95% CI: 97.7 - 100.0), a specificity of 100% (95% CI: 99.7 - 100.0), a positive predictive value of 100% (95% CI 99.7 -100.0) and a negative predictive value of 99.3% (95% CI 97.7 - 100.0).

DISCUSSION

Over the past few years, several RNA amplification methods have been developed to detect ZIKV, such as RT-PCR, real-

Table 2. Comparison of Zika virus detection limit by loop-mediated isothermal amplification method (ZIKV RT-LAMP) and reverse transcriptase polymerase chain reaction (qRT-PCR).

Amount of RNA (picograms)	RT-LAMP	qRT-PCR
7000	+	+
700	+	+
70	+	+
7	+	+
0.7	+	+
0.07	+	-
0.007	+	-
0.0007	+	-
0.00007	-	-

The ZIKV RT-LAMP method was 1,000 times more sensitive than the qRT-PCR.

time SYBR Green RT-PCR, and Taqman in qRT-PCR[7,11]. Although these methods have proven to be very reliable, they require standardized laboratory facilities, sophisticated equipment, and special instruments. In addition, well-trained personnel are needed to carry out the experiments. This study presents a novel and highly specific RT-LAMP method for the detection of ZIKV. This method provides advantages with respect to time, operation, feasibility, and cost. Some research groups have previously developed RT-LAMP-based methods to detect ZIKV showing very good results.

Calvert *et al.* reported an RT-LAMP with a sensitivity level 10 times higher than qRT-PCR[17]. However, the ZIKV RT-LAMP method showed a sensitivity level 1,000 times higher than qRT-PCR. Unlike Wang *et al.* who reported an RT-LAMP tested in simulated clinical samples showing a similar sensitivity to qRT-PCR[18], our ZIKV RT-LAMP was tested using 300 clinical samples, 150 of which were ZIKV.

Korosaki *et al.* reported about another RT-LAMP showing very interesting results, high sensitivity, high specificity, and was analyzed using serum, plasma, and urine samples[19]. However, the use of LAMP liquid reagent in the methodology can be very problematic when transporting and storing. ZIKV RT-LAMP solved this problem by introducing the use of lyophilized LAMP reagent. Song et al. reported an RT-LAMP method that is simple, easy to use, cost-effective, and applicable at the point of care, or near the patient's location[20]. It seems to have the same approach as our method, such as visual detection without the need for equipment.

The advantage of the ZIKV RT-LAMP method is that it has also proven useful with serum samples obtaining

Table 3. Validation of the method for the detection of Zika virus by loop mediated isothermal amplification (ZIKV RT-LAMP) using samples from other arboviruses.

Species*	ZIKV RT-LAMP vs qRT-PCR				
	Quantity	True positive	False positive	True negatives	False negatives
Zika	150	149	0	0	1
Dengue-1	15	-	0	15	-
Dengue-2	15	-	0	15	-
Dengue-3	15	-	0	15	-
Dengue-4	15	-	0	15	-
Chikungunya	20	-	0	20	-
Yellow fever	10	-	0	10	-
Oropouche	10	-	0	10	-
Mayaro	10	-	0	10	-
Negative to the previous ones	40	-	0	40	-
Total	300	149	0	150	1

*Species identification was previously performed by the gold standard reverse transcriptase polymerase chain reaction (qRT-PCR) method.

high values of sensitivity and specificity. These values are mainly due to the good design of the primers and the appropriate application of the methodology. The only limitation is that the LAMP methodology does not allow differential diagnosis in the same reaction, although this could be solved by designing primers for other arboviruses and placing them in additional tubes.

In conclusion, the ZIKV RT-LAMP method developed in this study allows a quick and reliable identification of ZIKV. Due to its low cost, practicality, and simple operation, they can be a good alternative for use even in primary healthcare facilities or local hospitals without complex laboratory equipment.

Authors' Contribution: EMO, CC, GGR, YW participated in the study conception, drawing up of the hypothesis, and design; EMO, GMP, MA, PE participated in data analysis and interpretation, article writing; EMO, CC, YW participated in the critical review of article

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Conflicts of Interest: All authors report no potential conflicts.

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