Recent molecular techniques for the diagnosis of Zika and Chikungunya infections: A systematic review

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ABSTRACT

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**A R T I C L E   I N F O**

**Zika virus (ZIKV)** and Chikungunya virus (CHIKV) are arboviruses that cause important viral diseases affecting the world population. Both viruses can produce remarkably similar clinical manifestations, co-circulate in a geographic region, and coinfections have been documented, thus making clinical diagnosis challenging. Therefore, it is urgent to have better molecular techniques that allow a differential, sensitive and rapid diagnosis from body fluid samples. This systematic review explores evidence in the literature regarding the advances in the molecular diagnosis of Zika and Chikungunya in humans, published from 2010 to March 2021. Four databases were consulted (Scopus, PubMed, Web of Science, and Embase) and a total of 31 studies were included according to the selection criteria. Our analysis highlights the need for standardization in the report and interpretation of new promising diagnostic methods. It also examines the benefits of new alternatives for the molecular diagnosis of these arboviruses, in contrast to established methods.

1. Introduction

ZIKV and CHIKV are arboviruses that cause important viral diseases that affect the world population. They are transmitted mainly by *Aedes aegypti* and *Aedes albopictus*; vectors that are established in tropical and subtropical regions. The impact on public health of these viruses is constantly increasing due to various factors such as population growth, urbanization, travel, and climate change, that facilitate transmission [1].

CHIKV is a member of the family *Togaviridae*, genus *Alphavirus*, and consists of a molecule of enveloped positive single-stranded RNA. Its genome encodes two open reading frames: the 5′ ORF is responsible for encoding the non-structural proteins of the virus (NSP) and the 3′ ORF encodes the capsid and envelope glycoproteins [2, 3, 4].

ZIKV is a single-stranded positive-sense RNA arbovirus belonging to the genus *Flavivirus* of the family *Flaviridae*. It contains a positive single-stranded genomic RNA that encodes a polyprotein that is processed into three structural proteins: the capsid (C), the membrane precursor (prM), and the envelope (E), and seven NSP [5].

According to the World Health Organization (WHO) in 2020, a total of 37,279 cases of chikungunya were reported in 11 countries in the Americas, 95% of the cases were notified by Brazil (35,447 cases). Bolivia and Brazil were the countries with the highest incidence rates, 13.3 and 17 cases per 100,000 inhabitants, respectively. In the same year, a total of 7,452 cases of Zika were reported. The countries with the most cases were: Brazil with 6,387 cases (86%), Bolivia with 537 cases (8.4%), and Guatemala with 133 cases (2%) [6].

These two arboviruses can produce a remarkably similar clinical picture, co-circulate in a geographic region, and coinfections have been documented, thus making clinical diagnosis difficult and, consequently, creating problems for proper management. Therefore, it is urgent to have better molecular techniques that allow a differential, sensitive and rapid diagnosis from body fluid samples.

Nucleic Acid Amplification Techniques (NAATs) are the most widely used molecular tests for virological diagnosis since they allow the detection of viral genome fragments. Various NAATs have been developed to improve performance and specificity, such as real-time Polymerase Chain Reaction (real-time PCR), where the amplified products are detected and quantified in each cycle of the reaction [7]. In nested PCR, the product of the first amplification is used as a template for a second amplification, providing high sensitivity and specificity [8]. In multiplex PCR, more than one sequence is simultaneously amplified, using different primer sets in the same reaction [9]. The NAAT that is most commonly used for the diagnosis of arbovirus infection is reverse transcriptase-polymerase chain reaction (RT-PCR) or its real-time version.
[10], where RNA is transcribed into complementary DNA using reverse transcriptase.

Progress is being made with other techniques such as Nucleic Acid Sequence-Based Amplification (NASBA), Transcription-Mediated Amplification (TMA), biosensors, and Retrotranscription Loop-Mediated Isothermal Amplification (RT-LAMP) [11]. RT-LAMP has gained strength in recent years due to its high specificity, low cost, and great speed to obtain results. It is a method that provides nucleic acid amplification at a constant temperature, allowing direct visualization of the reaction by measurement of turbidity, colorimetry, or fluorescence [12].

This systematic review explores the most recent evidence in the literature regarding the advances in the molecular diagnosis of Zika and chikungunya in humans, the current limitations and directions.

2. Material and methods

2.1. Search strategy and databases

A systematic review was performed to evaluate the novelties in the molecular diagnosis of Zika and chikungunya in humans, the search strategy was based on the recommendations of the PRISMA 2020 statement [13]. The search was done in four databases: Scopus, PubMed, Web of Science, and Embase, using the following terms: (“Zika” OR “chikungunya”) AND (“molecular tests” OR “molecular diagnostics” OR “molecular diagnosis”). The publication dates spanned from 2010 to March 2021.

2.2. Study selection

The present study only included original research articles, written in English, that focused on new techniques of molecular diagnosis in humans. Epidemiological studies, reviews, conference proceedings, theses, editorials/letters, meta-analyses, and case reports were excluded from this review. Tests in which viral antigens were detected using antibodies were not considered.

Once the inclusion and exclusion criteria were established (Table 1), the results were accounted for and were firstly screened by filtering the articles by title and abstract. Then, the results were imported and saved using Mendeley as reference manager, eliminating duplicated references using its built-in feature. The full text of the remaining articles was read to select the ones meeting the inclusion criteria.

2.3. Data extraction

The information contained in the selected articles was analyzed, taking into consideration the following relevant fields: basic information (title, authors, DOI, and publication year), information related to the virus (virus evaluated, strain detected, mutations associated with resistance or virulence), and type of molecular technique (genes evaluated, specificity and sensitivity, plexity, use of sequencing methods).

2.4. Risk of bias assessment

The risk of bias assessment was carried out using the Quality Assessment of Diagnostic Accuracy Studies-2 tool (QUADAS-2) [14]. QUADAS-2 is a tool used in systematic reviews to evaluate the risk of bias and applicability of primary diagnostic accuracy studies and consists of four key domains: patient selection, index test, reference standard, flow and timing. For each domain, a high, low, or unclear risk of bias was assigned, the results were a product of discussions between two of the reviewers (MCC-T and TO-C).

3. Results

3.1. Search results

The preliminary search in the databases identified a total of 567 articles (Scopus: 181, PubMed: 70, Web of Science: 97, and Embase: 219) which were added to the reference manager where 57 duplicate articles were removed. Following the selection process by title and abstract, 127 articles were eligible for full-text assessment. Finally, 96 articles were excluded by the criteria mentioned in Table 1, and 31 were selected for the present systematic review (Figure 1).

3.2. Study description

The studies included in the review and their characteristics are described in Table 2. The articles studied were published in a span of ten years, in which there has been an unsteady increase in the number of publications. Remarkably, the largest number of articles were published in 2020 (eight in total).

Fourteen articles describe the development of molecular techniques for the diagnosis of Zika, eleven for the diagnosis of chikungunya, and six describe the diagnosis of both arboviruses. Regarding the molecular technique, PCR is the most used, including its variations (fifteen articles), followed by RT-LAMP (nine articles). The number of studies performed with RT-LAMP increased each year, showing an interest to rely less on costly equipment such as thermocyclers. Additionally, six articles report the development of devices, sometimes powered by NAATs, for molecular diagnosis (Figure 2) [15-20].

The evaluation of the risk of bias in individual studies is shown in Figure 3. We did detect that the risk of bias for the patient selection criteria in nine articles is high due to the use of simulated clinical samples. Additionally, the reference standard used to validate the index test in some studies is unclear.

3.2.1. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR is a technique that allows the amplification of one or more RNA segments, either to detect them or use them in further assessments. Fourteen articles diagnosed Zika and chikungunya by this method.

Sharma et al. developed a variant of the technique to detect chikungunya, in which a competitor molecule containing an internal insert was generated, which provided strict control in the quantification process [21]. Chen et al. used a 2,7-diamino-1,8-naphthyridine derivative (DANP) in hairpin primers with labelled cytosine bulges to amplify the nsP2 region of the CHIKV genome, followed by measurement of the fluorescence emitted by the DANP-primer complex at the end of the PCR, which is inversely proportional to the viral load [22].

Nine articles simultaneously evaluated two or more genes, allowing for a differential diagnosis for both arboviruses and other viruses under study. These newly developed techniques showed very high specificity (100% in most cases) and minimal cross-reactivity. In some cases, it was even possible to detect phylogenetically diverse strains [23]. In addition, some studies evaluated the concordance of multiplex RT-PCR with a commercial uniplex assay, showing good consistency between them [24].

One of the studies designed new modifications to RT-PCR, including the development of a strategy to monitor the fluorescence signal of an L-DNA molecular beacon designed to indicate the ideal temperature for the reverse transcription hold step. The authors monitored L-DNA melting and annealing using a single optical channel, which made another

| Table 1. Inclusion and exclusion criteria used in this systematic review. |
|-----------------------------|-----------------------------|
| Inclusion criteria | Exclusion criteria |
| English | Any other language |
| Original research articles | Epidemiological studies, systematic reviews, conference proceedings, theses, editorials/letters, meta-analyses, or any other type of publication |
| Molecular diagnostic techniques | Serological diagnosis and other non-molecular techniques |
| Human studies | Vector-based studies |
channel available for multiplexing. They also developed highly sensitive
and specific primers and probes for real-time, multiplexed adaptive RT-
PCR for Zika, dengue, and chikungunya viruses together in a single
tube reaction [25].

Two studies performed quantitative reverse transcriptase PCR (RT-
qPCR) for the detection of Zika. Yang et al. developed an RT-qPCR
technique with primers that cover many strains as a solution for the
lack of genetic diversity coverage in ZIKV conventional primers and
probes [26]. Li et al. carried out a technique in which a RT-qPCR was
performed in clinical samples without the need for pre-treatment,
allowing for in situ detection of ZIKV [27]. Both articles showed high
specificity and sensitivity between 5 and 19 copies, however, when
scaling the tests to human blood, the sensitivity was much lower (1.9x10²
copies).

Giry et al. developed a RT-qPCR variant for the detection and
discrimination of Alphavirus, in which a TaqMan probe was used to target
the NSP4 gene [28]. The identified medically relevant viruses were
Chikungunya virus, Ross River virus, O’nyong Nyong virus, Sindbis virus,
and Semliki Forest virus.

3.2.2. Retrotranscription Loop-Mediated Isothermal Amplification (RT-
LAMP)
The RT-LAMP technique is a method that provides nucleic acid
amplification under isothermal conditions, using four or six primers
directed to specific target sequences, employing Bst DNA polymerase.

Wang et al. developed a one-step RT-LAMP portable platform for the
diagnosis of ZIKV using a metal bath, combined with a proteolysis
treatment capable of extracting enough RNA. The test specifically
detected ZIKV without cross-reactivity with other arboviruses or influ-
enza viruses, and can tolerate ZIKV genetic variation [29].

Kurosaki et al. formulated a RT-LAMP assay for the detection of ZIKV
by designing primers specific to Asian and African genotypes, using a
portable, battery-operated device. The assay did not show any cross-
reactions with other arboviruses or P. falciparum and did not yield
false-positive results [30]. In other instance, Guo et al. selected the NS5
gene as a target gene to detect ZIKV, as it showed high sensitivity and
specificity. They achieved the goal of obtaining quick accurate results by
completing the entire reaction process in 35 minutes by fluorescent
RT-LAMP [31].

Figure 1. Flow diagram of the study selection process. From: The PRISMA 2020 statement: an updated guideline for reporting systematic reviews [13].
Table 2. Characteristics of the studies included in the systematic review.

| Reference | Molecular test type          | Genes evaluated | Virus evaluated | Method specificity | Method sensitivity | Sequencing |
|-----------|-----------------------------|-----------------|-----------------|--------------------|--------------------|------------|
| [21]      | QC-RT-PCR                   | E1 gene         | Chikungunya     | It was verified with alpha and flavivirus, proving to be highly specific for CHIKV | 100 copies per reaction with a linear dynamic detection range of 102–1010 copies/reaction |            |
| [52]      | One-step duplex RT-PCR      | Does not specify | Chikungunya     | A clear and distinct band is evidenced at 354 bp for chikungunya and 511 bp for dengue, respectively. | 100 copies of RNA per reaction | X          |
| [22]      | DANP-Coupled Hairpin RT-PCR | nsP2 gene       | Chikungunya     | There were no false positives in 20 samples from uninfected patients. (100% specificity) | High sensitivity, picking up 21 of the 22 cases (95.5% sensitivity). |            |
| [39]      | A resequencing DNA microarray (RMA) | nsP4 gene or NS4B and NS5 genes | Chikungunya and dengue | Does not report | Does not report | X          |
| [55]      | SYBR Green-based Real-Time Multiplex RT-PCR | Does not specify | Chikungunya and dengue | High specificity (100%) when compared with conventional RT-PCR assay. | High sensitivity for DENV (100%) and CHIKV (95.8%) |            |
| [29]      | RT-LAMP                     | NS1             | Zika            | The primers recognize six to eight target sequences on a target gene, guaranteeing its strong specificity compared to PCR (100%) | 20 copies per reaction (4,000 copies/mL), and the estimated sensitivity of the assay for ZIKV reached 0.02 PFU per reaction (4 PFU/mL). | X          |
| [56]      | Single-reaction, Multiplex real-time RT-PCR | nsP2 gene       | Chikungunya and dengue | Amplification of separate DENV and CHIKV controls was detected in the appropriate channels without any cross-reaction, and both targets were consistently co-detected | Does not report |            |
| [57]      | One-step multiplex reverse transcriptase PCR | Does not specify | Chikungunya and dengue | 100% specificity for DENV and CHIKV | 95% sensitivity for DENV and 100% sensitivity for CHIKV |            |
| [15]      | Paper-based sensors and NASBA-GRISPR | Does not specify | Zika            | Dengue RNA sequences failed to activate the toehold switch sensors. | NASBA has exceptional sensitivity to low viral loads. |            |
| [54]      | Multiplex real-time RT-PCR  | nsP4 gene       | Zika, chikungunya and dengue | The triplex assay was 100% specific and did not amplify any of the other viruses tested. | The 95% LOD by the triplex assay was 15 copies per reaction for DENV-1 and less than 10 copies/reaction for all other viruses. | X          |
| [35]      | RT-LAMP, LFA                | Envelope protein gene | Zika            | Amplification was not observed in any of the RNA preparations from the DENV and YFV strains, or gene fragments of WNV. The assay displayed high specificity for ZIKVs | Notably, the existence of even a single copy of ZIKV RNA could be detected with the LFA. |            |
| [26]      | One-step qRT-PCR            | ZIKV envelope (E) gene | Zika            | Two exchanged bases in the target DNA significantly reduce the opening of FQH. This assay is specific for the differentiation between closely related pathogens. | Two exchanged bases in the target DNA significantly reduce the opening of FQH. This assay is specific for the differentiation between closely related pathogens. |            |
| [16]      | FQH-based DNA detection.    | Does not specify | Zika            | The RT-LAMP assay did not show any false-positive results compared to RT-qPCR. | Ten copies of the RNA standards were detected per reaction. 100% clinical sensitivity compared to RT-qPCR |            |
| [30]      | RT-LAMP                     | E gene          | Zika            | The RT-LAMP assay did not show any false-positive results compared to RT-qPCR. | Ten copies of the RNA standards were detected per reaction. 100% clinical sensitivity compared to RT-qPCR |            |
| [28]      | Real-time RT-PCR            | nsP4            | Chikungunya     | Unrelated viruses were not detected with their pan-Alphavirus assay | They obtained a LOD at 40 copies per reaction. 100% clinical sensitivity relative to CHIKV RT-PCR assay |            |
| [24]      | Multiplex Real-time RT-PCR  | Envelope (E) gene and 3’ UTR gene | Zika, chikungunya and dengue | No cross-amplification was observed among these viruses. Exclusivity was performed with an extensive panel of pathogens. No amplification signal was observed | 95% LOD (copies per reaction): DENV-1 (19), DENV-2 (13), DENV-3 (24), DENV-4 (36), ZIKV-Africa (15), ZIKV-Asian (9), CHIKV (13) |            |
| [31]      | RT-LAMP                     | NS5             | Zika            | Does not cross-react with bacteria | The LOD can reach 3.3 ng/μL. |            |
| [23]      | One-step multiplex real-time RT-PCR | ZIKV NS5 - 3’ UTR, CHIKV nsP1 | Zika and Chikungunya | Highly specific for targeted viruses showing no amplification of a variety of other flaviviruses. The assay was able to detect the phylogenetically diverse strains of ZIKV and CHIKV. | The lowest limit of detection of the multiplex assay was 1 and 0.5 PFU for ZIKV and CHIKV, respectively. |            |
| [58]      | Pentaplex RT-qPCR assay     | 3’ UTR (ZIKV and DENV), NSP2 (CHIKV) and NS5 (WNV genomes) | Zika, Dengue, Chikungunya, West Nile | Results only detected the specific sequence, and no cross-reaction was observed. | LOD of 100 RNA copies per reaction for ZIKV in serum or urine, 100 RNA copies per reaction for DENV in serum, and 10 RNA copies per reaction for CHIKV and CHIKV, WNV and 95.10% for ZIKV |            |

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In contrast with the previously mentioned studies, Estrela et al. presented a faster ZIKV detection method by performing RT-LAMP directly on raw samples with an exceptionally low limit of detection. The assay was validated using a panel of 51 human serum samples confirming a 100% agreement [32]. Hayashida et al. developed a protocol involving drying all the RT-LAMP reagents in a single reaction tube. With this system, RT-LAMP was performed for CHIKV and the amplification products were used as templates for MinION sequencing to identify viral genotypes [33]. Similarly, Saechue et al. developed a dry RT-LAMP system using trehalose as a powerful protector, which prevented the enzyme from suffering damage during freeze drying. Additionally, they designed new RT-LAMP primers targeting the CHIKV E1 region and used fluorescent and colorimetric indicators [34].

Lee et al. developed a method for detecting ZIKV using RT-LAMP and a lateral flow assay (LFA). Additionally, RT-LAMP conditions were optimized by adjusting the concentration of the MgCl₂ and dNTP mixture to prevent non-target amplification [35]. Bui et al. developed a one-step RT-LAMP assay to detect ZIKV lineages trying to distinguish between African and Asian lineages [36]. The Asian lineage is of specific importance as it is associated to Guillain-Barré syndrome and microcephaly in newborns.

Sharma et al. developed an automated microfluidic chip-based RT-LAMP assay that combines isolation, purification, and amplification steps in the same platform and enables visual detection of ZIKV from human plasma in 40 minutes [19]. The entire ZIKV diagnostic procedure is performed within a uniquely designed, economical, and disposable microfluidic chip. Along the same line, Lee et al. developed a multiplexed

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**Table 2 (continued)**

| Reference | Molecular test type | Genes evaluated | Virus evaluated | Method specificity | Method sensitivity | Sequencing |
|-----------|---------------------|-----------------|-----------------|-------------------|-------------------|------------|
| [27]      | Direct reverse-transcription quantitative PCR (dirRT-qPCR) | NS5 non-structural protein gene | Zika            | The specificity of the assay was not affected by the co-presence of other infections, such as viral or bacterial infections, along with the ZIKV infection. 100% clinical specificity relative to standard RT-qPCR. | Detection limits of ZIKV RNA were 19 copies/μL, while in simulated whole blood it was 1.9 × 10⁶ copies/μL. 100% clinical sensitivity relative to standard RT-qPCR. | Does not specify |
| [25]      | Multiplexed Adaptive RT-PCR | Does not specify | Zika, Dengue and Chikungunya | All reactions amplified within one cycle for each virus RNA in combination with each or with both of the other targets. | Detection limit of 5 copies per reaction of Zika or chikungunya and 50 copies of dengue per reaction. | Does not specify |
| [32]      | RT-LAMP | Does not specify | Zika            | Fluorescence increased when ZIKV RNA was used as the template and not with DENV samples or healthy human specimen controls, with 100% clinical specificity. | RT-LAMP reactions allowed detection of amplicons in reactions starting at 10⁻³ copies of RNA per reaction (10 μL), 100% clinical sensitivity relative to RT-PCR. | Does not specify |
| [33]      | Dried RT-LAMP system (CHIKV-CZC-LAMP) | E1 region of CHIKV genome | Chikungunya | No positive reaction was observed in the 4 endemic healthy control RNA and serum samples, demonstrating 100% specificity | Analytical sensitivity of the assay was <50 PFU per reaction. 70% (95% CI: 0.51–0.84) sensitivity for RNA samples and 58% (95% CI: 0.39–0.75) sensitivity for serum samples. | Does not specify |
| [20]      | Chitosan-modified capillary assist, microfluidic-based in situ PCR method | Does not specify | Zika            | Does not report | 2.5 × 10⁷ genome equivalents per mL | Does not specify |
| [19]      | Automated microfluidic chip-based LAMP assay. | 429 bp from the GenBank (KX702400), was chosen as the target. | Zika            | No increase in the fluorescent signal was observed in the negative control reaction carrying HIV. | The lowest limit of detection observed was 102 RNA copies per reaction | Does not specify |
| [18]      | Lab-on-paper for all-in-one molecular diagnostics | Does not specify | Zika, Dengue and Chikungunya | Does not report | The detection limits were 5–5,000 copies of ZIKV. | Does not specify |
| [37]      | EEM fluorescence spectroscopy with chemometric techniques | Does not specify | Dengue and Chikungunya | Models (specificity): n-PLSDA (Uninfected 66.66%; DENV 83.33%; CHIKV 100%; PARAFAC-LDA (Uninfected 66.66%; DENV and CHIKV 100%; PARAFAC-QDA (Uninfected, DENV, and CHIKV 100%) | Models (sensitivity): n-PLSDA (Uninfected 87.50%; DENV 80%; CHIKV 75.0%; PARAFAC-LDA (Uninfected and CHIKV 100%; DENV 83.33%; PARAFAC-QDA (Uninfected, DENV, and CHIKV 100%) | Does not specify |
| [34]      | Liquid RT-LAMP and Dry RT-LAMP system | E1 gene | Chikungunya | The system selectively detected CHIKV but no other viruses even at the highest viral load dilutions | The sensitivity of the RT-LAMP system was 104 times lower in mice serum than in the viral culture. The RT-LAMP system failed to detect CHIKV in the serum from infected patients | Does not specify |
| [17]      | Peptide nucleic acid (PNA)/nano-sized graphene oxide (GO)-based biosensor combined with loop-mediated isothermal amplification (LAMP). | Does not specify | Zika and Dengue | Discriminated between Zika and Dengue, and distinguished between different Dengue serotypes | The sensitivity of overall detection assays for ZIKV, DENV was in a range of 2.1 × 10²–5.1 × 10¹ FFU/mL | Does not specify |
| [38]      | CRISPR-Cas CRISPR-Cas12 | Does not specify | Zika and Dengue | Assays only detected the specific sequence, and no cross-reaction was observed | The test showed a clinical sensitivity and positive predictive value of 100% | Does not specify |
| [36]      | RT-LAMP RT-LAMP-based SNPs typing | Does not specify | Zika            | Zika was specifically detected and sorted into African and Asian lineages. | Detection limit ranging from 0.17 FFU/mL to 2.3 × 10⁵ FFU/mL. | Does not specify |
detection strategy using a nano-sized graphene oxide (GO) based fluorometric biosensor with peptide nucleic acid (PNA) probes, combined with RT-LAMP [17]. Their data demonstrated the detection ability of the PNA/GO biosensor for ZIKV and DENV S1-24 in sera.

3.2.3. Unconventional molecular diagnostic methods

Ochmann et al. developed a nucleic acid detection method that avoids molecular amplification using a direct physical fluorescence amplification mechanism through a plasmonic nanoparticle that acts as an optical antenna (Figure 4) [16]. The unique scaffolding properties of DNA origami were exploited to anchor a target-specific fluorescence-quenching hairpin (FQH) that detects nucleic acids (the research used RNA or synthetic Zika virus DNA). Furthermore, they showed how the modularity offered by DNA nanotechnology allows multiplexing by incorporating orthogonal fluorescent tags for the simultaneous detection of various sequences. This work allowed them to glimpse on the possibilities of DNA nanotechnology to report the presence of different pathogens with simplified detection devices.

Pardee et al. worked on a technique for molecular diagnosis based on the design, assembly, and validation of cell-free toehold switch RNA sensors for the detection of ZIKV (Figure 5) [15]. They linked isothermal RNA amplification with NASBA, and when combined with a novel CRISPR-Cas9 module, their sensors discriminated between viral strains with a single base resolution. These diagnostic sensors allow for the
detection of target sequences in the femtomolar range, aligning the sensitivity of the sensor with viral concentrations in patients. An optimized sensor development platform could provide a generalizable method for rapid response during emerging outbreaks.

Zhu et al. created a device for the rapid extraction and detection of Zika virus [20]. The system consists of four modified chitosan capillaries integrated into a microfluidic chip that interact with Zika virus and performs an in situ PCR amplification, acting as a smartphone-based point of care (POC) device (Figure 6). All detection processes, including sample lysis, ZIKV RNA enrichment, and reverse transcription-polymerase chain reaction (RT-PCR) were accomplished on the microfluidic chip. For ZIKV RNA obtained from saliva samples, the detection of this device was within the limit of around 50 Transduction Units (TU)/mL, which indicates that the method is reliable to detect viremia in infected patients.

Santos et al. conducted a study that demonstrated the potential of excitation-emission matrix spectroscopy coupled to multi-way analysis to sort the sera from clinical samples into non-infected and arbovirus-infected by either DENV or CHIKV. The results found in this study were encouraging but further studies must be designed to associate this information with the biochemical structures present in viruses, or with intrinsic variations in virus-cell interactions [37].

Curti et al. implemented a CRISPR-Cas12a system with a reverse transcription step that made it possible to detect Zika and dengue sequences, with a diagnosis time of fewer than two hours and at attomolar concentrations. This method was validated using clinical samples from patients infected with dengue. The presence of the virus was detected employing a fluorescent signal and using a lateral flow test, demonstrating the ability of the CRISPR-Cas12a system to detect nucleic acid targets with a shortened processing time (Figure 7) [38]. It was also demonstrated that it is possible to use this system in lateral flow assays, avoiding the use of additional instrumentation.

Berthet et al. [39] developed a panel of viral pathogens called PID2-RMA using resequencing microarray technology (RMA), suitable for the identification and characterization of arboviruses in biological samples. The PID2-RMA contains a set of 126 viral sequences and demonstrated efficacy in identifying three clinically important mosquito-borne RNA viruses: DENV, WNV (West Nile virus), and CHIKV. This technique would be particularly suitable for emergency cases, where the identification of the pathogen is crucial for efficient patient care, particularly for those living in arbovirus endemic areas. Furthermore, the mixing of all viruses in a single human blood sample did not interfere with the sensitivity of PID2-RMA. However, whatever microarray technology is used, the technique is always limited when there are low pathogen loads in the samples or when new variants arise with diverging sequences.

Seok et al. showed an all-in-one paper laboratory for the molecular diagnosis (LAMDA) of ZIKV, DENV, and CHIKV in human sera. The entire processing that involves sampling, RNA extraction, nucleic acid amplification, and detection; is operated on a paper chip, using dry chemicals (Figure 8). Fluorescent RT-LAMP was employed to simultaneously detect ZIKV, DENV, and CHIKV in the all-in-one paper chip in 60 minutes at 65 °C. The use of a portable hand warmer to maintain incubation at 65 °C for
the paper chip box and a smartphone to measure the fluorescence signal makes this technology a POC device. Although some challenges remain, LAMDA provides a portable, low-cost, easy-to-use, sensitive, and specific NAAT with great potential for POC testing [18].

4. Discussion

The distribution of Zika and Chikungunya cases reported for the Americas for the time period 2008–2020 [6] indicates that the publication dates selected for the search strategy did cover the 2014 and 2015 epidemics caused by chikungunya virus, and the 2016 epidemic caused by Zika virus. This information correlates with the percentage of studies published as of this date. Of the 31 selected articles that reported novel molecular techniques for diagnosing both viruses, 87% were published between 2015 and 2020.

Diagnostic methods based on RT-PCR have been used to detect RNA in infectious agents, and are considered the most sensitive and specific techniques for their detection [40]. Although RT-PCR is considered the gold standard test for the detection of Zika and chikungunya, several characteristics place it at a disadvantage compared to other methodologies. This technique requires a step for the extraction of the genetic material before the test and, besides, there is a high cost associated with the thermal cyclers making it challenging to implement in remote areas and developing countries. The risks of RNA degradation during the transportation and processing of the samples are high and imply an inversion in low temperature equipment [30]. It is necessary to develop new techniques that solve these problems while maintaining the sensitivity and specificity of the Zika and chikungunya diagnostic.

Various molecular diagnostic techniques have been developed in recent years. RT-LAMP is a nucleic acid amplification method that offers multiple benefits such as efficacy, sensitivity, and ease of use [12, 41]. Research carried out in recent years has made it possible to identify various advantages offered by RT-LAMP over conventional methodologies. First, four primers are specifically designed to target six specific regions of the genome under study and if even one primer does not match, the reaction is not carried out which gives high specificity to this technique. As a second advantage, RT-LAMP offers high sensitivity, with detection limits of even 10 copies of nucleic acid or less, as is the case with some articles presented in this review [30, 42]. In addition, it is a technique that functions at a constant temperature, achievable with affordable equipment, such as a water bath or incubator. Complex clinical samples – blood, serum, urine, and saliva – can be directly analyzed avoiding the extraction step because Bst polymerase is an enzyme tolerant to inhibitory molecules. Finally, the technique has alternative detection methods, such as the measurement of turbidity, fluorescence, color, and ion concentrations.

These advantages show that the technique is suitable for the early diagnosis of various diseases, even in the absence of symptoms. Furthermore, the time required for detection in this assay is much shorter than in RT-PCR [43, 44]. Despite all the advantages, there are limitations; for example, possible contamination by aerosols that can lead to false positives [45, 46]. Likewise, as this technique requires more primers, the probability of forming dimers increases, which can have a negative effect on its specificity [31] Lee et al. optimized the concentration of dNTP as an alternative to eliminate non-target amplification in reactions.

CRISPR-Cas systems are also used in the detection of these arboviruses. This technique has revolutionized genetic engineering. These systems represent the most powerful weapon of defense in bacteria and archaea, acting against invading foreign nucleic acids [47]. Based on their function, these systems have been used for the detection of nucleic acids in attomolar concentrations [48], placing it as a highly sensitive technique. The CRISPR-Cas systems can also detect DNA and RNA with a unique base mismatch specificity [49].

Other advantages offered by CRISPR-Cas systems are the simplicity for the development of the technique, and its high resolution that allows the detection of single base variations [38]. However, CRISPR-Cas systems may require several steps of sample manipulation, increasing the probability of bias. Additionally, the commercial information about the method is still incipient causing a lack of knowledge in critical aspects.
such as enzyme stability, inhibitors, and reaction enhancers. Such conditions may be counterproductive when scaling up the method for clinical diagnosis.

Although alternative methods such as RT-LAMP and biosensors with CRISPR-Cas systems, among others, seek to reduce costs by avoiding the use of thermal cyclers, the benefits provided by this equipment must be considered. Thermal cyclers confer quality and reliability to the results and their efficacy has been many times tested, even by commercial companies, using clinical samples.

Some variations have been implemented to RT-qPCR to avoid the use of real-time PCR instruments. For instance, Chen et al. evaluated the absence of fluorescence at the endpoint of a DANP-coupled hairpin RT-
PCR, as indicator of the virus presence using a fluorometer. However, strict quality control cannot be carried out since the absence of fluorescence may also be caused by factors such as a minimal amount of primer or primer dimerization [22]. Although fluorometers lower costs, real-time PCR instruments provide more reliable clinical diagnostics by allowing nucleic acid amplification to be followed step by step.

The new molecular diagnostic methodologies seek to implement nucleic acid detection methods at the point of care that are low-cost and fast enough to allow early detection of pathogens. However, when implementing these methods, where generally all the detection steps are integrated in the same system, their sensitivity and specificity need to be deeply analyzed as there may be hindrances related to clinical samples, such as reaction inhibition, host nucleic acid background, cross reactivity with other pathogens, and low amounts of the target in the sample [16, 38].

The accuracy of a diagnostic test is given by its sensitivity—the proportion of infected individuals that test positive—and its specificity—the proportion of uninfected individuals that test negative. These aspects are normally reported based on samples collected by the authors, sometimes analyzed in parallel using already established methods. Such approach may only show a relative or overvalued accuracy, without a well-defined detection limit and a considerable number of false positives. Cohen et al [50] compiled the results from external quality evaluations of RNA virus PCR assays for fifty laboratories, revealing false-positive rates for chikungunya in a range of 1.9% to 8.1% in 2007 and 2014 respectively, and 2.8% for Zika in 2016. This information shows the need for internal validation tests and proficiency assessments by independent testing organizations to evaluate how different sample conditions may affect the test sensitivity, such as poor quality of the sample, the phase of the disease in which it is taken, poor handling or shipping conditions [51]. In addition, these assessments would allow a smooth transition to a clinical environment, by specifying which types of sample could be analyzed by the test, the stability required to avoid virus degradation [33], and measuring the clinical sensitivity and specificity, beyond focusing only in the analytical detection limit and cross-reactions using microbial isolates [15, 16, 17, 19, 21, 23, 24, 25, 26, 29, 31, 34, 36, 52, 53, 54].

5. Conclusion

This systematic review provided literature evidence of the advances in the molecular diagnosis of Zika and chikungunya. The diagnostics have been based in NAATs and, more recently in CRISPR-Cas, sequencing, and colorimetric or fluorometric biosensors. In general, there has been a tendency towards the design of more simple, cost-effective, and portable techniques, independent of sophisticated equipment and able to detect different arboviruses. However, it would also be essential to establish diagnostic techniques capable of detecting more virulent strains that may even be part of genomic surveillance programs. Furthermore, this research highlights the need for uniformity in the evaluation of specificity and sensitivity using clinical samples or independent proficiency assessments.

Declarations

Author contribution statement

María C. Cardona-Trujillo, Tatiana Ocampo-Cárdenas, Fredy A. Tabares-Villa, and Augusto Zuluaga-Vélez: Conceived and designed the experiments;Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Juan Carlos Sepúlveda-Arias: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest’s statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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