J Bras Patol Med Lab. 2021; 57: 1-9.

10.5935/1676-2444.20210048

The use of real time PCR for arboviruses diagnostics: integrative review

O uso de PCR em tempo real em diagnósticos de arboviroses: revisão integrativa

Christiane O. L. Licínio; Flávio M. Ayres

Universidade Estadual de Goiás, Anápolis, Goiás, Brazil.

ABSTRACT

Arboviruses are viral diseases transmitted by arthropods (arthropod-borne virus). Standing out dengue, zika virus, and chikungunya among the emergent and re-emergent arboviruses in recent years around the world. The similarity between the symptoms makes the clinical diagnosis ineffective, making difficult the prophylactic and preventive measures of new outbreaks. Molecular diagnosis using the real-time polymerase chain reaction (PCR) technique is one of the ways to diagnose such diseases. In this study, the literature on the diagnosis of arboviruses was compiled and evaluated. The objective was to answer the guiding question: Is the real-time PCR methodology effective in the diagnosis of arboviruses? Scientific articles of free access were searched in the databases Pubmed (50 articles) and Scielo (107 articles), between 2014 and 2019. The selection was done through the inclusion and exclusion criteria, only 20 articles remained. Among them, 85% cross-sectional studies, 10% systematic reviews, and 5% case studies. The period of publications was 50% in 2017, 35% in 2016, and 5% in 2014, 2015 e 2019, each. Regarding the viruses treated in the articles, 25% researched dengue and the same percentage for chikungunya, 20% researched about zika virus. The efficacy of the molecular diagnosis was published in 21% of the articles (sensitivity and specificity), 53% highlighted the limit of detection, 70% highlighted the absence of cross-reactions, and 80% highlighted the differentiation between viruses.

Key words: molecular probes techniques; dengue virus; zika virus; chikungunya virus.

RESUMO

*As arboviroses são doenças virais transmitidas por artrópodes (*arthropod-borne virus*). Destacam-se dengue, vírus da zica e chikungunya entre as arboviroses emergentes e reemergentes nos últimos anos em todo o mundo. A semelhança dos sintomas dessas infecções faz com que o diagnóstico clínico seja ineficaz, dificultando medidas profiláticas e preventivas para novos surtos. O diagnóstico molecular por meio da técnica de reação em cadeia da polimerase (PCR) em tempo real é uma das formas de diagnosticar tais doenças. Neste estudo, foi compilada e avaliada a literatura sobre o diagnóstico das arboviroses. Nosso objetivo foi responder a uma pergunta norteadora: a metodologia PCR em tempo real é eficaz no diagnóstico das arboviroses? Foram pesquisados artigos científicos de livre acesso nos bancos de dados Pubmed (50 artigos) e Scielo (107 artigos), entre 2014 e 2019. A seleção foi realizada por meio dos critérios de inclusão e exclusão, restando apenas 20 artigos. Entre estes, 85% eram estudos transversais, 10%, revisões sistemáticas e 5%, estudos de caso. O período das publicações foi de 50% em 2017; 35% em 2016; e 5% em 2014, 2015 e 2019, cada. A respeito dos vírus tratados nos artigos, 25% dos estudos pesquisaram sobre dengue; 25%, sobre chikungunya e 20%, sobre o vírus da zica. A eficácia do diagnóstico molecular foi publicada em 21% dos artigos (sensibilidade e especificidade); 53% destacaram o limite de detecção; 70%, a ausência de reações cruzadas; e 80%, a diferenciação entre os vírus.*

Unitermos: *técnicas de diagnóstico molecular; vírus da dengue; vírus da zica; vírus da chikungunya.*

First submission on 03/20/20; last submission on 03/27/20; accepted for publication on 06/26/20; published on 10/20/21

RESUMEN

*Las arbovirosis son enfermedades virales transmitidas por artrópodos (*arthropod-borne virus*). Dengue, zica y chikungunya se destacan entre los arbovirus emergentes y reemergentes en los últimos años en todo el mundo. La similitud de los síntomas de estas infecciones hace que el diagnóstico clínico sea ineficaz, dificultando las medidas profilácticas y preventivas para nuevos brotes. El diagnóstico molecular mediante la técnica de reacción en cadena de la polimerasa (PCR) en tiempo real es una de las formas de diagnosticar esas enfermedades. En este estudio se recopiló y evaluó la literatura sobre el diagnóstico de arbovirosis. Nuestro objetivo era responder a una pregunta orientadora: ¿la metodología de PCR en tiempo real es eficaz para diagnosticar arbovirosis? Se buscaron artículos científicos de acceso abierto en las bases de datos Pubmed (50 artículos) y Scielo (107 artículos), entre 2014 y 2019. La selección se realizó utilizando los criterios de inclusión y exclusión, quedando solo 20 artículos. Entre estos, el 85% fueron estudios transversales, el 10% fueron revisiones sistemáticas y el 5% fueron estudios de casos. El período de publicaciones fue del 50% en 2017; 35% en 2016; y 5% en 2014, 2015 y 2019, cada. En cuanto a los virus tratados en los artículos, el 25% de los estudios investigaron sobre el dengue; el 25% el chikungunya y el 20% el virus del Zica. La efectividad del diagnóstico molecular se publicó en el 21% de los artículos (sensibilidad y especificidad); el 53% destacó el límite de detección; 70%, ausencia de reacciones cruzadas; y el 80%, la diferenciación entre virus.*

Palabras clave: *técnicas de diagnóstico molecular; vírus del dengue; vírus del zika; vírus del chikungunya.*

INTRODUCTION

Arbovirus is a nomenclature used to indicate a grouping of arthropod-borne viruses. Mosquitoes and ticks are examples of arthropods capable of transmitting, through the bite, viruses belonging primarily to three families: *Togaviridae*, *Flaviviridae,* and *Bunyaviridae*. The most important genus among these three families is Flavivirus, which has four members with great epidemiological importance: dengue (DENV), zika virus (ZIKV), yellow fever (YFV), and West Nile virus (WNV). From the *Togaviridae* family, the Alphavirus genus stands out, whose member of greatest epidemiological importance is chikungunya (CHIKV). All of these genera are enveloped, positive-sense, single-stranded ribonucleic acid (RNA) viruses[1].

These pathogens are responsible for major disease outbreaks around the world, especially in the last 20 years. The fact that they are transmitted by insects, which are capable of spreading over a large geographic extension, contributed to the occurrence of epidemics[2]. There is no presence of arboviruses only on the Antarctic continent[3, 4]. Vectors, vertebrate hosts, and climatic conditions are the main factors for arboviruses to spread so quickly[1]. In addition, the high capacity for mutation and adaptation also influence the occurrence of large outbreaks[5].

Recent climate change, large agglomerations due to uncontrolled urbanization, precarious sanitary conditions, and the great human movement between continents contribute to the proliferation of arboviruses[1-3, 6]. Therefore, these viruses are the protagonists of emerging and re-emerging diseases, which caused a significant number of deaths and economically impacted several countries over the years[3]. Viruses are co-circulating in several countries and, in some cases, have the same vector[7].

Initially, arboviruses present as an acute febrile illness, followed by symptoms of arthralgia, myalgia, and thrombocytopenia[5, 8]. This makes the clinical differential diagnosis precarious, that is, the onset of these symptoms only is inefficient to identify the pathogen causing the disease. Consequently, laboratory diagnosis, with high sensitivity and specificity, is essential for this approach[8]. Although initial symptoms are common, some cases progress to complications after infection. As an example, hemorrhagic fever in cases of DENV, microcephaly, and Guillain-Barré syndrome in cases of ZIKV[5]. Thus, the differentiation between arboviruses is important for patient management in order to avoid complications, as well as to assist in taking preventive measures to control the spread of the disease[9].

The laboratory diagnosis for arboviruses can be performed in two ways: indirectly, through the investigation of antibodies in the infected patient's blood, or directly, through the investigation of the pathogen in the blood and other body fluids[1]. The most common diagnosis for Flaviviruses is made by the enzyme-linked immunosorbent assay (Elisa), which screens for class M immunoglobulin (IgM) antibodies for the early stages of the disease. However, the use of this methodology leads to numerous cross-reactions between the various arboviruses[10]; therefore, the main method for diagnosing arboviruses in the early stage of

the disease is the reverse transcription reaction followed by the polymerase chain reaction (RT-PCR)[9].

Molecular diagnoses, such as RT-PCR and real-time PCR, are very sensitive and specific, as they reduce the occurrence of cross-reactivity and identify pathogens in the early stages of the disease[10]. Several biological matrices can be used, such as urine, semen, amniotic fluid, and saliva, besides whole blood, serum, and plasma[1]. The advantage of real-time PCR compared to traditional PCR is the quantification of the amplified material simultaneously with the amplification of the tested genetic material. This process reduces the time spent on the reaction and the possibility of cross contamination. In RNA amplifications, which have low stability in the molecule, speed reduces the incidence of false negative results[11].

Considering the emergence and re-emergence of the DENV, CHIKV, ZIKV, and YF arboviruses in recent years and the need to identify and differentiate these viruses, the objective of this review was to compile and analyze the scientific literature regarding the diagnosis of arboviruses. The focus of the research was limited to the effectiveness of the molecular diagnosis used to identify and differentiate viruses, in particular on sensitivity, specificity, occurrence of cross-reactivity, and limit of detection.

METHODOLOGY

This article is an integrative review that was carried out following the steps below: 1. selection and identification of the theme – formulation of the guiding question; 2. establishment of inclusion and exclusion criteria; 3. definition of information (articles) through research in databases; 4. evaluation and categorization of information; 5. interpretation of the results obtained; and 6. presentation of results.

The guiding question was: Is the real-time PCR methodology effective in the diagnosis of arboviruses? The PubMed and Scielo databases were used to search for open access scientific articles, in English and Portuguese. The period of publication was delimited from January 2014 to July 2019 in order to select the most current and relevant articles on the topic, since this subject has been more disseminated from 2014 on. MESH and its equivalent in Portuguese, DECS, were used to find the descriptors "real-time PCR", "arbovirus", and "molecular diagnosis". The Boolean operator "AND" was used in the PubMed database search, and "OR" in the Scielo database. There was no restriction on the design of the article.

Publications were selected by title and abstract. Repeated articles and texts that did not contain information about the diagnosis of the disease or that were not about arboviruses were excluded. The exclusion criteria also applied to articles with approaches outside the aforementioned arboviruses.

The selection of articles was carried out in February 2020. Fifty articles in the PubMed database and 107 articles in the Scielo database were found (we did not find duplicate articles). After applying the inclusion and exclusion criteria, we selected 32 articles, however, nine were not open access and three did not meet the objectives. Therefore, the research was completed with 20 articles. **Figure** shows the flow of steps carried out in the research.

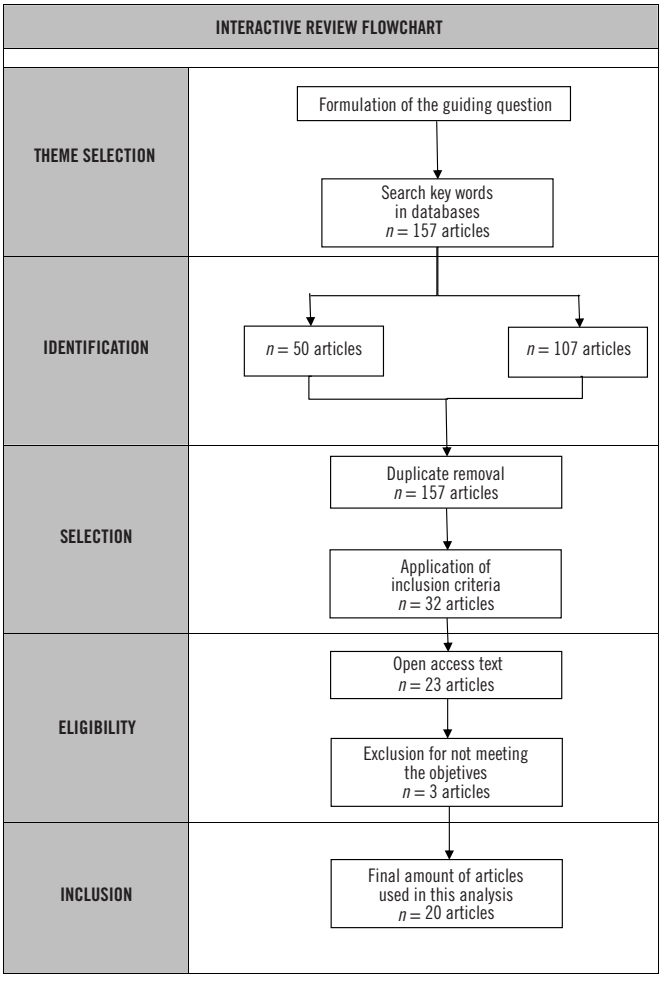

FIGURE – *Flowchart of search and selection steps of the analyzed articles*

RESULTS

Of the 20 articles selected, 85% were cross-sectional studies, 10% systematic reviews, and 5% were case studies. Most articles

were written in 2017 (50%) and 2016 (35%). 2019, 2015, and 2014 had the same number of publications (5%), as described in **Table 1**.

Regarding the viruses of the study, **Table 2** shows that 25% of the articles reported exclusively on DENV, the same percentage on CHIKV, and 20% on ZIKV. The percentage of articles that searched for all three viruses at the same time was also 20%. The search on DENV and CHIKV, and DENV and ZIKV in the same article was 5% each disease group. No article mentioned the YF.

Table 3 summarizes the results of the studies regarding the parameters investigated for the effectiveness of the test in question. 21%of the articles reported numerical data for sensitivity and specificity. The limit of detection was published in 53% of the articles. Differentiation between viruses was mentioned in 80% of the selected texts, and the absence of cross-reactivity in 70%.

DISCUSSION

Arboviruses have become a public health problem in almost every country in the world. Rapid spread, availability of vectors, lack of effective treatment, and prevention make epidemics frequent and increasingly virulent. The adaptive capacity of viruses as a result of genetic variations greatly contributes to their emergence in new geographic regions and to the frequency of outbreaks in regions where they are already established[12].

Arboviruses were much debated in Brazil due to the occurrence of ZIKV and CHIKV outbreaks in 2015 and the recurrence of DENV for several years. There were few publications on the diagnosis of arboviruses between 2014 and 2015, before the outbreak, and between 2018 and 2019, after the outbreak. Most publications were between 2016 and 2017. It was also possible to verify that articles from cross-sectional studies are the majority, which characterizes the predominance of observational articles.

TABLE 1 – Articles selected during the stages of integrative review search

Article	Authors	Article title	Research design	Year of publication
1	Alva-Urcia *et al*.[1]	Emerging and reemerging arboviruses: a new threat in Eastern Peru	Cross-sectional study	2017
2	Kurosaki *et al*.[2]	Development and evaluation of a rapid molecular diagnostic test for zika virus infection by reverse transcription loop-mediated isothermal amplification	Cross-sectional study	2017
3	Giry *et al*.[3]	Improved detection of genus-specific Alphavirus using a generic TaqMan® assay	Cross-sectional study	2017
4	Edwards *et al*.[4]	Analytical and clinical performance of a chikungunya qRT-PCR for Central and South America	Cross-sectional study	2017
5	Giry *et al*.[5]	Simultaneous detection of chikungunya virus, dengue virus and human pathogenic Leptospira genomes using a multiplex TaqMan® assay	Cross-sectional study	2017
6	Luo *et al*.[6]	Laboratory and molecular characterization of dengue viruses in a 2014 outbreak in Guangfo region, Southern China	Cross-sectional study	2017
7	Fortuna *et al*.[7]	Imported arboviral infections in Italy, July 2014-October 2015: a National Reference Laboratory report	Cross-sectional study	2017
8	Eppes *et al*.[8]	Testing for zika virus infection in pregnancy: key concepts to deal with an emerging epidemic	Systematic review	2017
9	Corman *et al*.[9]	Assay optimization for molecular detection of zika virus	Cross-sectional study	2016
10	Johnson *et al*.[10]	Laboratory diagnosis of chikungunya virus infections and commercial sources for diagnostic assays	Cross-sectional study	2016
11	Patel *et al*.[11]	A field-deployable reverse transcription recombinase polymerase amplification assay for rapid detection of the chikungunya virus	Cross-sectional study	2016
12	Pessôa *et al*.[12]	Investigation into an outbreak of dengue-like illness in Pernambuco, Brazil, revealed a cocirculation of zika, chikungunya, and dengue virus type 1	Cross-sectional study	2016
13	Chen *et al*.[13]	Development and evaluation of a sybr green-based real-time multiplex RT-PCR assay for simultaneous detection and serotyping of dengue and chikungunya viruses	Cross-sectional study	2016
14	Abd El Wahed *et al*.[14]	Recombinase polymerase amplification assay for rapid diagnostics of dengue infection	Cross-sectional study	2015
15	Xavier *et al*.[15]	Clinical and laboratory diagnosis of Zika fever: an update	Systematic review	2017
16	Souza *et al*.[16]	Clinical and laboratory diagnosis of congenital zika virus syndrome and diaphragmatic unilateral palsy: case report	Case study	2016
17	Acosta *et al*.[17]	False-negative dengue cases in Roraima, Brazil: an approach regarding the high number of negative results by NS1 Ag kits	Cross-sectional study	2014
18	Romeiro *et al*.[18]	Evaluation and optimization of SYBR Green real-time reverse transcription polymerase chain reaction as a tool for diagnosis of the Flavivirus genus in Brazil	Cross-sectional study	2016
19	Galo *et al*.[19]	Development of in-house serological methods for diagnosis and surveillance of chikungunya	Cross-sectional study	2017
20	Slavov *et al*.[20]	Simultaneous zika and dengue serotype-4 viral detection and isolation from a donor plasma unit	Cross-sectional study	2019

TABLE 2 – Objectives of selected articles, samples used in the studies and brief summary of the main results

Article	Objective	Samples	Target virus of the research	Main results
1	Evaluate the prevalence of DENV, OROV, CHIKV, MAYV and ZIKV in patients with acute fever disease in the city of Puerto Maldonado (Peru)	139 human serum samples	DENV CHIKV ZIKV	41 (29.5%) positive for arboviruses; 13 (9.35%) positive for CHIKV; nine (6.48%) positive for DENV; seven (5.03%) positive for ZIKV. The differentiation between arboviruses is precarious by clinic only
2	Develop and evaluate a rapid molecular diagnostic test for ZIKV with the RT-LAMP methodology	120 suspect samples, 90 serum/plasma and 99 urine	ZIKV	100% agreement between RT-LAMP and qRT-PCR, the limit of detection of qRT-PCR being the most sensitive
3	Implement a new molecular method for detecting Alphavirus	Virus strains obtained from various laboratories	CHIKV	Positivity in CHIKV samples. LoD greater than the reference test in the same methodology. Twelve virus species were tested and there was no cross-reactivity
4	Develop and validate a qRT-PCR assay capable of detecting all CHIKV lineages	RNA from virus culture and serum samples from Guatemala and Ecuador in July 2015	CHIKV	Effective assay, mainly the strain circulating in South America. Compared to CDC USA PCR, 98% sensitivity and 100% specificity
5	Implement a syndromic approach based on the use of a multiplex qPCR assay to facilitate rapid diagnosis of dengue-like syndromes in Reunion Island	Virus strains from various laboratories and positive human plasma	CHIKV DENV	Evaluation and accuracy of the assay were satisfactory for CHIKV and DENV, offering reliable answers. It has been shown to be a cheaper option than simple tests for the same pathogens
6	Investigate the usefulness of simple laboratory tests as predictive markers of confirmed DENV diagnoses, as well as to analyze the genotype and phylogenetics of the circulating strain in the dengue epidemic in Guang-Dong in 2014	1044 patients: 875 confirmed DENV cases (862 positive by NS1 and 13 by PCR) and 169 negative	DENV	Simple laboratory tests (leukopenia, thrombocytopenia, aminotransferases and activated thromboplastin time) are useful markers in the diagnosis of DENV
7	Describe the results of analyzes of infections by arboviruses imported from Italy carried out by the National Reference Laboratory for Arboviruses (NRLA) at the Italian National Institute of Health from July 2014 to October 2015	Inpatient serum samples ($n = 180$)	DENV CHIKV ZIKV	Greater number of cases of DENV. There was an increase in CHIKV cases, and the first case of ZIKV was detected. In the latter, the PRNT technique was decisive for identification; the virus was negative in serology and PCR techniques (which proved to be sensitive and specific). However, only for a short period of time, limited to viremia in the body
8	Review laboratory testing methodology to explore virus presence and immune response	Blood, serum, urine, amniotic fluid, CSF, saliva, semen, breast milk and vaginal mucosa	ZIKV	Viremia level is low in the serum, from two to 10 days, which makes molecular diagnosis difficult and increases the number of false negatives. The case study demonstrated permanence of the virus in red blood cells for 81 days, suggesting that the use of whole blood may increase sensitivity
9	Examine the diagnostic performance of real-time PCR for detection of ZIKV	Serum and urine from individuals who travelled to Brazil, Dominican Republic and Suriname between 2015 and 2016	ZIKV	Low level viremia has been shown to cause false negatives. This study found no difference between blood and urine viremias, but suggests the use of the two samples combined. Assays with NS1 are more specific and sensitive, while assays with NS3 and NS5 have low sensitivity and difficulty in specific probes, respectively
10	Describe the results found by CDC USA on the efficiency of diagnostic kits for CHIKV	Serum and plasma	CHIKV	For PCR, the NS2 target is more sensitive, but there is no publication on its validation. Other methodologies were effective and had no cross-reactions
11	Develop and evaluate a real-time PCR assay to potentially be a point of care diagnostic tool for detecting CHIKV	Human serum and plasma	CHIKV	The assay was shown to have a clinical sensitivity and specificity of 100%, a low limit of detection and only a cross-reaction with a little-known virus (O'nyong'nyong). With the use of a differentiated primer, the cross-reaction was extinguished
12	Investigate and identify viral etiology and advise health authorities on implementing control measures to contain an outbreak	77 human plasma samples, collected at Hospital Severino de Souto Siqueira, in Recife, PE, between 25 and 31 May 2015	DENV CHIKV ZIKV	31 (40.2%) patients with ZIKV infection; nine (11.7%) patients infected with DENV; one (1.3%) patient positive for CHIKV IgM, but negative in the PCR test. Coinfection by ZIKV/DENV was proven in 2.6% of patients. The study highlights the need for differentiation between viruses to improve health control measures

Cont. →

→ *Cont.*

13	Develop a specific, sensitive and robust assay in the RT-LAMP methodology for diagnosis and differentiation between DENV serotypes 1-4	190 serum samples	DENV serotypes 1-4	RT-LAMP results were satisfactory; this methodology was more effective than traditional PCR and real-time PCR. Results (RT-LAMP, RT-PCR, qRT-PCR): sensitivity 98.9%, 84.2% and 90.5%, respectively; specificity 100%, 93% and 100%, respectively
14	Develop two RT-RPA assays to detect DENV 1-4	Human samples from outbreaks in Senegal and Thailand	DENV serotypes 1-4	Results (RT-RPA and qRT-PCR): sensitivity 98% RNA Thailand and 72% RNA Senegal; 98% RNA Thailand and 94.4% RNA Senegal. Specificity 100% and 100%, respectively
15	Update the clinical and laboratory diagnosis of fever by VZIK	blood, saliva and urine. Amniotic fluid, CSF, placenta and umbilical cord in cases of neonatal infection	VZIK	qPCR detects acute infection within seven days of symptom onset. Blood is less sensitive than urine (15 days) and saliva. Third day of manifestation the best time for testing. In neonates with clinical symptoms and negative PCR, perform serology
16	Report a case of unilateral diaphragmatic paralysis in a neonate with a confirmed diagnosis of congenital zika by amniotic fluid examination using RT-PCR and by CSF serology	Amniotic fluid and CSF	DENV CHIKV ZIKV	Positive RT-PCR in amniotic fluid at week 29 of gestation. Negative in maternal and newborn serum after delivery (acute infection during pregnancy). IgM in newborn serum confirms intrauterine infection (IgM does not cross placental barrier)
17	Suggest that NS1 Elisa tests have low sensitivity and produce false negative results and cross reactions with other arboviruses	150 human serum samples which tested DENV negative by Elisa	DENV	33 Elisa negative samples were positive for qRT-PCR, ie 22% false negatives were truly positive; 75% were from DENV-4. Correlation with other studies in which secondary DENV infection produces false negatives in NS1 kits
18	Evaluate and optimize a diagnostic test using the qRT-PCR methodology for the Flavivirus genus	410 serum samples with acute fever, DENV negative by RT-PCR	DENV	qRT-PCR is more sensitive and specific than traditional PCR, in addition to allowing virus quantification. There was no amplification of other Flavivirus, indicating that there is no cross-reaction
19	Develop and evaluate serological methods for diagnosis and research of CHIKV in Nicaragua	260 acute and convalescent serum samples	CHIKV	qRT-PCR was used as the gold standard. Seven PCR positive samples were serologically negative; four PCR negative and serology positive samples may indicate serology cross-reactivity. Low sensitivity of serology in the acute phase, being high for PCR in the same phase
20	Report the detection of DENV and ZIKV by qRT-PCR in a pre-symptomatic blood donor, in Ribeirão Preto, São Paulo	A blood sample (plasma) from the donor	DENV ZIKV	Detection of ZIKV infection concurrently with DENV infection. 13 million copies/ml of ZIKV and 5 million copies/ml of DENV-4 were found in the same blood donor plasma

DENV: dengue; OROV: oropouche virus; CHIKV: chikungunya; MAYV: mayaro virus; ZIKV: zika virus; RT-LAMP: reverse transcription loop-mediated isothermal amplification; qRT-PCR: reverse transcriptase reaction followed by polymerase chain reaction; CDC: Centers for Disease Control and Prevention; IgM: immunoglobulin class M; RT-RPA: reverse transcriptase polymerase reaction; RNA: ribonucleic acid; CSF: cerebrospinal fluid.

TABLE 3 – Effectiveness of real-time PCR assays performed on articles selected in the integrative review

Article	Sensitivity	Limit of detection	Specificity	Cut-off value	Differentiation between viruses	Cross-reaction
1	HSe	NI	HSp	NI	Yes	No
2	NI	10 cp/assay	NI	< 35	Yes	No
3	AS	40 cp/assay	HSp	< 20.96	Yes	No
4	98.4%	19.6 cp/assay	100%	< 37	Yes	No
5	HSe	NI	HSp	< 36	Yes	No
6	NI	NI	NI	< 38	NI	NI
7	HSe	NI	HSp	NI	Yes	No
8	NI	NI	NI	NI	NI	NI
9	HSe	10 cp/assay	HSp	NI	Yes	No
10	HSe	5.3 cp/assay	HSp	NI	NI	NI
11	100%	80 cp/assay	100%	< 36	Yes	No
12	HSe	100 cp/ml	HSp	< 40	Yes	No
13	90.5%	100 cp/ml	100%	NI	Yes	No
14	94.4%-98%	10 cp/µl	100%	< 38	Yes	No
15	HSe	NI	HSp	NI	NI	NI

Cont. →

→ *Cont.*

16	NI	NI	NI	NI	Yes	NI
17	NI	NI	NI	< 39	Yes	NI
18	HSe	100 cp/ml	HSp	NI	Yes	No
19	NI	NI	NI	NI	Yes	No
20	NI	NI	NI	NI	Yes	No

PCR: polymerase chain reaction; HSe: high sensitivity (numeric value not informed); NI: not informed; HSp: high specificity (numeric value not informed); cp: virus copies.

Among arboviruses, the Flaviviruses (DENV, ZIKV, and YF) and Alphaviruses (CHIKV) stand out as the pathogens that cause acute febrile illness, with evolution to neurological febrile or hemorrhagic complications[12]. As they are cocirculating in the same geographic area, the differential diagnosis is even more difficult[13]. The consequences of this cocirculation are not yet fully known and can be a worsening factor in the evolution of infections[12]. There are reports of co-infection in the same patient with DENV-ZIKV, DENV-CHIKV or ZIKV-CHIKV[14].

In addition to cases of coinfection, there is a concern with reinfection, especially in cases of DENV. Among arboviruses, it is the disease with the highest number of cases and the highest morbidity and mortality. The virus has four serotypes, each of which can cause a distinct infection[1]. At each reinfection by a different serotype, the antibodies are not able to neutralize the virus and cause disease amplification mediated by antibodies [antibody-dependent enhancement (ADE)][12, 15]. Therefore, a second DENV infection is even more serious than the first, with high-level viremia and several inflammatory markers released into the bloodstream[12].

Many researches are focused on identifying whether, as in the case of a DENV reinfection, cocirculation and co-infection with ZIKV can cause ADE in secondary infections by this pathogen[16]. There are many similarities between DENV and ZIKV, which causes a large number of cross-reactions in antibodies. Some researchers have raised the possibility that ZIKV is a fifth serotype of DENV[17], as their E protein genetic sequence is shared in a proportion between 54% and 59%[15]. Stettler *et al*. (2016)[18] showed that there was cross-reactivity of the immune system between a previous DENV infection and a secondary ZIKV infection in the antibodies against the viral envelope domains I and II (EDI/II), causing a low neutralization of the ZIKV.

Two publications refer to co-infections. One of them reports the case of a 28-year-old female patient who was diagnosed with co-infection with DENV and CHIKV, in Fortaleza, Ceará, Brazil. A severe hematologic complication was proven in the patient due to the coinfection; initially, she presented only the nonspecific symptoms of arboviruses (fever, myalgia, and arthralgia). The patient developed acquired thrombotic thrombocytopenic purpura (TTP), antibody-mediated thrombocytopenia, a serious complication of CHIKV infection[19]. The other publication, from 2019, reports the case of a pre-symptomatic blood donor. Three days after the blood donation, she showed characteristic symptoms and returned to the donation site. In the investigation, RNA of ZIKV and DENV were detected simultaneously in blood plasma collected during donation[20].

The articles selected in this integrative review had objectives such as product development, comparative analysis between methodologies and, less frequently, the review of studies on the diagnosis of arboviruses. In 20% of them, the three most epidemiologically important viruses of arboviruses were investigated, as seen in Table 2. Article 12 identified 2.6% of cases of ZIKV-DENV coinfection in a hospital in Recife, Brazil, during the 2015 outbreak, highlighting the need to distinguish the viruses to improve sanitary controls.

The importance of diagnosing arboviruses is not restricted to differentiating which agent is the cause of the infection, it is also crucial in studies investigating severe cases of diseases, such as in the associations between ZIKV and neurological disorders. In addition, diagnosis is essential in seroprevalence studies and surveillance of new epidemics[17], as well as in alternative transmission routes[21].

Molecular diagnosis of arboviruses is a widely used tool in the acute stage of infection, in which viremia is at its apex. There are several methodologies for direct pathogen research, and the real-time PCR technique demonstrates many advantages compared to others available on the market. This is due to the ease of execution of the methodology, the lower value of reagents and equipment, and the lower risk of contamination, both for the operator and the sample[9].

The real-time PCR technique is a variation of traditional PCR; uses fluorescent probes that monitor the amplification of genetic material throughout the reaction. This process is called real time, as amplification and quantification occur simultaneously[22]. It is a fast, specific, and sensitive technique that can detect more than one pathogen in the same reaction (multiplex qPCR), as long as there are different fluorescent dyes between the reagents[23].

The primers used for amplification are designed according to the pathogen being investigated. The development of these primers

with the help of bioinformatics has increased the sensitivity and specificity of the reagents. This is due to greater identification of genomic regions of pathogens, differentiating them from other individuals of their family or genus[23].

The parameters used to determine the effectiveness of an assay are sensitivity and specificity. Sensitivity refers to the test being truly positive, that is, when the individual actually has the investigated disease and the test is positive. Specificity is related to the fact that the test is truly negative when there is no disease and the result is negative. The low sensitivity of an assay produces false-negative results, and the low specificity produces false-positive results[24].

The limit of detection and occurrence of cross-reactivity are derived from these parameters. The first parameter refers to the minimum amount of an analyte, in this case, the viral RNA – capable of detecting a particular sample. The articles analyzed showed that the real-time PCR technique is very sensitive. For each gene [deoxyribonucleic acid (DNA)] or gene expression (RNA) detection, there is a limit of detection that varies from less than 100 to 5.3 copies/sample run; this last amount is considered very low and borderline in the detection of viral RNA[25].

Real-time PCR primers are designed according to the genome of the virus under investigation. In differentiating between viruses, there is the use of distinct fluorescent reagents in different primers, highlighting each virus. In cross-reactions, the primers bind in different regions of the virus, allowing the distinction between phylogenetically similar viruses, such as ZIKV and DENV.

CONCLUSION

Real-time PCR methodology is effective in diagnosing arboviruses. It is able to differentiate DENV, ZIKV, and CHIKV viruses, with low occurrence of cross-reactivity and low nonspecific reaction. Additionally, the technique is able to identify the pathogen even at low-level viremia, which is important for early diagnosis of the disease. Diagnosis is fast, sensitive, and specific, which makes it a highly reliable diagnostic tool.

ACKNOWLEDGMENTS

This work was carried out with the support of the Fundação de Amparo à Pesquisa do Estado de Goiás (Goias Research Foundation) – process number 201810267000572.

REFERENCES

1. Vasudevan RS. Dengue and zika: control and antiviral treatment strategies [Internet]. Vol. 1062. 2018. Available at: http://www.springer.com/series/5584.

2. Lorenz C, Aguiar BS, Azevedo TS, Chiaravalloti Neto F, Suesdek L. Impact of environmental factors on neglected emerging arboviral diseases. PLoS Negl Trop Dis. 2017; 1-19.

3. Li X, Gao X, Fu S, et al. Arboviruses and their related infections in China: a comprehensive field and laboratory investigation over the last 3 decades. Rev Med Virol. 2017; 1-21.

4. Lopes N, Nozawa C, Linhares REC. Características gerais e epidemiologia dos arbovírus emergentes no Brasil. Rev Pan-Amazônica Saúde [Internet]. 2014; 5(3): 55-64. Available at: http://scielo.iec.pa.gov.br/scielo.php?script=sci_arttext&pid=S2176-62232014000300007&lng=en&nrm=iso&tlng=en.

5. Alva-Urcia C, Aguilar-Luis MA, Palomares-Reyes C, et al. Emerging and reemerging arboviruses: a new threat in Eastern Peru. PLoS One. 2017; 12(11): 1-13.

6. Gould E, Pettersson J, Higgs S, Charrel R, Lamballerie X. Emerging arboviruses: why today? 2017; 4(June): 1-13.

7. Edwards T, del Carmen Castillo Signor L, Williams C, et al. Analytical and clinical performance of a Chikungunya qRT-PCR for Central and South America. Diagn Microbiol Infect Dis [Internet]. 2017; 89(1): 35-9. Available at: http://dx.doi.org/10.1016/j.diagmicrobio.2017.06.001.

8. Giry C, Roquebert B, Li-Pat-Yuen G, Gasque P, Jaffar-Bandjee MC. Simultaneous detection of chikungunya virus, dengue virus and human pathogenic Leptospira genomes using a multiplex TaqMan® assay. BMC Microbiol. 2017; 17(1): 1-10.

9. Romeiro MF, de Souza WM, Tolardo AL, et al. Evaluation and optimization of SYBR green real-time reverse transcription polymerase chain reaction as a tool for diagnosis of the flavivirus genus in Brazil. Rev Soc Bras Med Trop. 2016; 49(3): 279-85.

10. Hu SF, Li M, Zhong LL, et al. Development of reverse-transcription loop-mediated isothermal amplification assay for rapid detection and differentiation of dengue virus serotypes 1-4. BMC Microbiol [Internet]. 2015; 15(1): 1-15. Available at: http://dx.doi.org/10.1186/s12866-015-0595-1.

11. Arya M, Shergill IS, Williamson M, Gommersall L, Arya N, Patel HRH. Basic principles of real-time quantitative PCR. Expert Rev Mol Diagn. 2005; 5(2): 209-19.

12. Donalisio MR, Freitas ARR, Freitas R, Von Zuben APB. Arboviroses emergentes no Brasil: desafios para a clínica e implicações para a saúde pública. Rev Saúde Pública. 2017; 51: 30.

13. Giry C, Roquebert B, Li-Pat-Yuen G, Gasque P, Jaffar-Bandjee MC. Improved detection of genus-specific Alphavirus using a generic TaqMan® assay. BMC Microbiol. 2017; 17(1): 1-9.

14. Chaves BA, Orfano AS, Nogueira PM, et al. Coinfection with zika virus (ZIKV) and dengue virus results in preferential ZIKV transmission by vector bite to vertebrate host. 2018; 218: 563-71.

15. Roth C, Delgado FG, Simon-Lorière E, Sakuntabhai A. Immune responses to dengue and Zika viroses — guidance for T cell vaccine development. Int J Environ Res Public Health [Internet]. 2018; 15(2): 1-12. Available at: http://www.mdpi.com/1660-4601/15/2/385.

16. Felix AC, Souza NCS, Figueiredo WM, et al. Cross reactivity of commercial anti-dengue immunoassays in patients with acute Zika virus infection. J Med Virol [Internet]. 2017; 89(8): 1477-9. Available at: https://doi.org/10.1002/jmv.24789.

17. Balmaseda A, Stettler K, Medialdea-Carrera R, et al. Antibody-based assay discriminates Zika virus infection from other flaviviruses. Proc Natl Acad Sci [Internet]. 2017; 201704984. Available at: http://www.pnas.org/lookup/doi/10.1073/pnas.1704984114.

18. Stettler K, Beltramello M, Espinosa DA, et al. Specificity, cross-reactivity, and function of antibodies elicited by Zika virus infection. Science. 2016; 353(6301): 823-6.

19. Bastos MLA, Araújo RMO, Oliveira DS, Cavalcante ANM, Júnior GBS. Thrombotic thrombocytopenic purpura associated with dengue and chikungunya virus coinfection: case report during an epidemic period. [Internet]. Rev Inst Med Trop São Paulo. 2018; 1-4. Available at: https://doi.org/10.1016/j.orcp.2018.05.003.

20. Slavov S, Ferreira F, Rodrigues E, Gomes R, Covas D, Kashima S. Simultaneous zika and dengue serotype-4 viral detection and isolation from a donor plasma unit. J Vector Borne Dis. 2019; 56(2): 166-9.

21. Corman VM, Rasche A, Baronti C, et al. Assay optimization for molecular detection of Zika virus. Bull World Health Organ. 2016; 94(12): 880-92.

22. Kim T, Vo D, Bigot P, et al. Evaluation of a real-time PCR assay for malaria diagnosis in patients from Vietnam and in returned travellers. Trans R Soc Trop Med Hyg. 2007; 101(5): 422-8.

23. Nunes ARD, Alves BEB, Pereira HWB, et al. Improved reverse transcription-polymerase chain reaction assay for the detection of flaviviruses with semi-nested primers for discrimination between dengue virus serotypes and Zika virus. Memórias Instituto Oswaldo Cruz. 2018; 113(5): 1-9.

24. Kawamura T. Interpretação de um teste sob a visão epidemiológica. Eficiência de um teste. Arq Bras Cardiol. 2002; 79(2): 437-41.

25. Esposito DLA, Fonseca BAL. Sensitivity and detection of chikungunya viral genetic material using several PCR-based approaches. Rev Soc Bras Med Trop. 2017; 50(4): 465-9.

CORRESPONDING AUTHOR

Christiane Oliveira Lima Licínio ID 0000-0003-3114-4595
e-mail: chrislima28@hotmail.com