



A systematic review of molecular diagnostic methods for the detection of arboviruses in clinical specimens in Brazil and the importance of a differential diagnosis

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Abstract

Arboviruses (Arthropod-borne viruses) compose a large group of zoonotic viruses that have complex cycles and often cause diseases in humans. This study was aimed at performing a systematic review of molecular diagnostic methods for the detection of arboviruses in clinical samples from Brazil to emphasize the importance of a differential diagnosis. Articles on arbovirus diagnostic methods were searched in databases using descriptors and selection criteria. A total of 19 articles were found that described techniques that may be used for the differential diagnosis of arboviruses. RT-PCR, nested RT-PCR and real-time RT-PCR were the main methods. The samples were collected from the Brazilian Amazon and the state of São Paulo for the diagnosis of dengue, Oropouche fever, yellow fever, Saint Louis encephalitis and Mayaro virus disease. Classical diagnostic methods were rarely used. In addition, molecular methods are not yet fully standardized because several methods did not detect arboviruses. Furthermore, a diagnosis that is based only on clinical and epidemiological data would be premature. Therefore, new entomological research and new differential molecular methods should be performed for the possible isolation of these unknown viruses to contribute to the diagnosis of arboviruses. Thus, additional research should be conducted because these diseases are emerging and reemerging in several countries.

Keywords: Arbovirus, systematic review, differential diagnosis, RT-PCR, arboviruses in Brazil

Introduction

Arboviruses (Arthropod-borne viruses) compose a large group of zoonotic viruses that infect hematophagous arthropods and have complex cycles. Arboviruses are commonly transmitted to humans mainly by the bite of mosquitoes and less frequently by ticks, which results in disease. Arboviruses are classified into the *Togaviridae* (genus *Alphavirus*), *Flaviviridae* (genus *Flavivirus*), *Bunyaviridae* (genus *Orthobunyavirus* and *Phlebovirus*) and *Reoviridae* families. Most arboviruses have a single-strand RNA genome with spherical morphology and a diameter that ranges from 45-120 nm [1,2]. The dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), Saint Louis encephalitis virus (SLEV), Venezuelan equine encephalitis virus (VEEV), Eastern and Western equine encephalitis viruses, West Nile virus (WNV) and Oropouche virus (OROV) are included in this group of viruses. Arboviruses are clinically important, and at least 2.5 billion people live in areas at risk for dengue [3-5].

Currently, many of the diseases that are caused by arboviruses are considered to be emerging and reemerging. In South America, the DENV, YFV, SLEV, OROV, Mayaro virus (MAYV) and Rocio virus (ROCV) are the main causes of emerging and reemerging diseases. These diseases are an important public health problem, as they cause large epidemics and result in increased financial costs that are associated with diagnosis and treatment. In addition, the mortality rates of several of these diseases are considerable. Despite the worldwide distribution of arboviruses, countries in tropical and subtropical areas with vast

forest reserves and diverse fauna represent environments that are more conducive to the ecology of arboviruses. More than 33% of the territory (3 million km²) in Brazil consists of natural ecosystems that provide ideal conditions for the development and dissemination of arboviruses [2,5,6].

In Brazil, still were isolated or conducted serological surveys in humans, animals and mosquitoes the following virus shown in (Table 1) [7-17]. In addition, the Chikungunya virus, which originates from Southeast Asia and the East African coast, was isolated; however, all of the cases were imported [18-20]. Most of these infections were asymptomatic. A symptomatic infection is usually accompanied by clinical signs of acute fever, encephalitis and hemorrhagic fever [1,21-24].

The diagnosis of arboviral diseases can be difficult due to the following factors: a clinical similarity to other diseases; the presence of clinically asymptomatic or oligosymptomatic disease; difficult access to reference laboratories that can perform a differential diagnosis; and phylogenetic cross-reactions that can occur between serological tests, especially in endemic regions, such as the city of Manaus, Brazil, where dengue can be easily confused with yellow fever (YF), MAYV fever, malaria or Oropouche fever [25,26]. In addition to Manaus, concomitant cases of SLEV infection and dengue have occurred in other states, such as São Paulo. Consequently, many of these infections have been neglected, excluding dengue, and few epidemiological studies have been conducted in the country to understand the distribution of these infections. Moreover,

Table 1. Arbovirus isolated in the Brazil.

Family	Genus	Virus	Viral Properties
<i>Togaviridae</i>	<i>Alphavirus</i>	Venezuelan/East/West equine encephalitis virus*, Mucambo*, Trocara, Pixuna*, Chikungunya*, Mayaro*, Una*, Aura*	Single-stranded RNA and the positive sense, size of 9.7-11.8 kb, spherical (70 nm diameter), envelope, 3 or 4 major structural polypeptides, which 2 are glycosylated. Replication: cytoplasm; Virion maturation: sprouting through the cell membranes of the host.
<i>Flaviviridae</i>	<i>Flavivirus</i>	Dengue*, SLEV*, Bussaquara*, Ilhéus*, Iguapé*, Rocio*, Cacipacore*, YFV*	Single-stranded RNA and the positive sense, size of 11-21 kb, spherical (45-60 nm diameter), envelope, 3 or 4 major structural polypeptides, which 2 are glycosylated. Replication: cytoplasm; Virion maturation: within the endoplasmic reticulum.
<i>Bunyaviridae</i>	<i>Orthobunyavirus</i> <i>Phlebovirus</i>	Oropouche*, Maguari*, Tacaiuma*, Xingu*, Apeu*, Caraparú*, Itaqui*, Guama*, Catu*, Guaroa*, Oriboca*, Nepuyo*, Murutucu*, Marituba*, Turlock, Tucunduba*, Melao Alenquer*, Candiru*, Morumbi*, Serra Norte*	Single-stranded RNA with 3 segments, negative sense, or both senses, size of 11-21 kb, spherical (80-120 nm de diameter). Virion contains one viral RNA dependent RNA polymerase, 4 major polypeptides and wrap. Replication: cytoplasm; Virion maturation: sprouting in the smooth membranes of Golgi system.

*Arboviruses associated with human disease. The more important arboviruses causing human disease are DENV, YFV, OROV, MAYV, SLEV and ROCV [24].

the differential diagnosis of arboviral infections in Brazil has been accomplished in few laboratories and mainly for dengue. In addition, false positives and false negatives can occur, thus "masking" the epidemiological data [1,11].

An early and accurate diagnosis is critical for successful treatment. The reverse transcriptase-PCR (RT-PCR) method and the variants of this test can meet these requirements because the method is highly sensitive and specific, which allows for the identification of virus in small samples and serotyping to detect the virulence of viral strains [27,28]. Few studies have reviewed the criteria, the methods and the application of molecular methods to diagnose the infections that are caused by arboviruses. This study was aimed at performing a systematic review of the scientific literature on the differential molecular diagnostic methods for the detection of arboviruses in clinical samples from Brazil.

Methods

This study was a systematic review, conducted to identify, analyze and summarize the results of independent studies that were based on the molecular diagnosis of infections that are caused by arboviruses. Commonly, a review of studies can be divided into a narrative, integrative and systematic review in which a statistical test can be applied, called a meta-analysis, which is useful for evaluating treatment studies [29,30]. In

this study, the researchers developed a research protocol with inclusion and exclusion criteria for the studies that were analyzed and a definition of the outcomes of interest. Therefore, our research strategy was divided into the following phases: (1) the definition of the descriptors, (2) the selection of the data sources, (3) the analysis and data extraction of the articles and (4) the presentation of the results.

Definition of the descriptors-Phase 1

To meet the objectives of this research, descriptors were defined, which consisted of words or a set of words that represented the subject of the study. The following terms were used in English and in Portuguese for this study: PCR combined with arbovirus/arboviruses; association between the terms molecular diagnostics, differential diagnosis, dengue, SLEV, Venezuelan/East/West equine encephalitis virus, arbovirus in Brazil, YF, West Nile fever, OROV, MAYV, ROCV, Una virus, Bussaquara virus, Pixuna virus, Iguape virus, Ilheus virus, Trocara virus, Cacipacore virus (CPCV), Melão virus, Aura virus and Chikungunya virus.

Selection of data sources-Phase 2

The following databases were searched: Literatura Latino-Americana e do Caribe em Ciências da Saúde (LILACS), PubMed, Scientific Electronic Library Online (SciELO) and Google Scholar. All of these databases are indexed by scientific journals. The SciELO includes paper, online newspapers with approximately 290 journals from Brazil, Cuba, Chile and other countries in Latin America, which are provided at no cost. The LILACS database comprises a cooperative network Virtual Health Library (VHL), which includes literature on the health sciences and is published in Latin America and the Caribbean.

These databases were searched from February 2012 to September 2012 using selected keywords, and manuscripts that were published between 1990 and 2012 were evaluated. Reviews and experimental studies that involved animals were excluded from the study.

Analysis and data extraction from the articles-Phase 3

After phases 1 and 2, studies were selected based on the abstract and evaluated independently by two researchers. In this phase, we evaluated the origin, the methodological design and the clarity of the articles.

The researchers met regularly to discuss the studies that were found in the databases (LILACS, SciELO, PubMed and Google Scholar), and this phase of the systematic review consisted of consensus meetings. The following variables were extracted from the studies: the type of the virus that was isolated from clinical specimens; the molecular techniques that were used; the ability of the differential diagnostic methods to accurately diagnose arboviral diseases; the current knowledge of the topic and the possible identification of gaps in knowledge. The data from the articles were carefully analyzed [29-31].

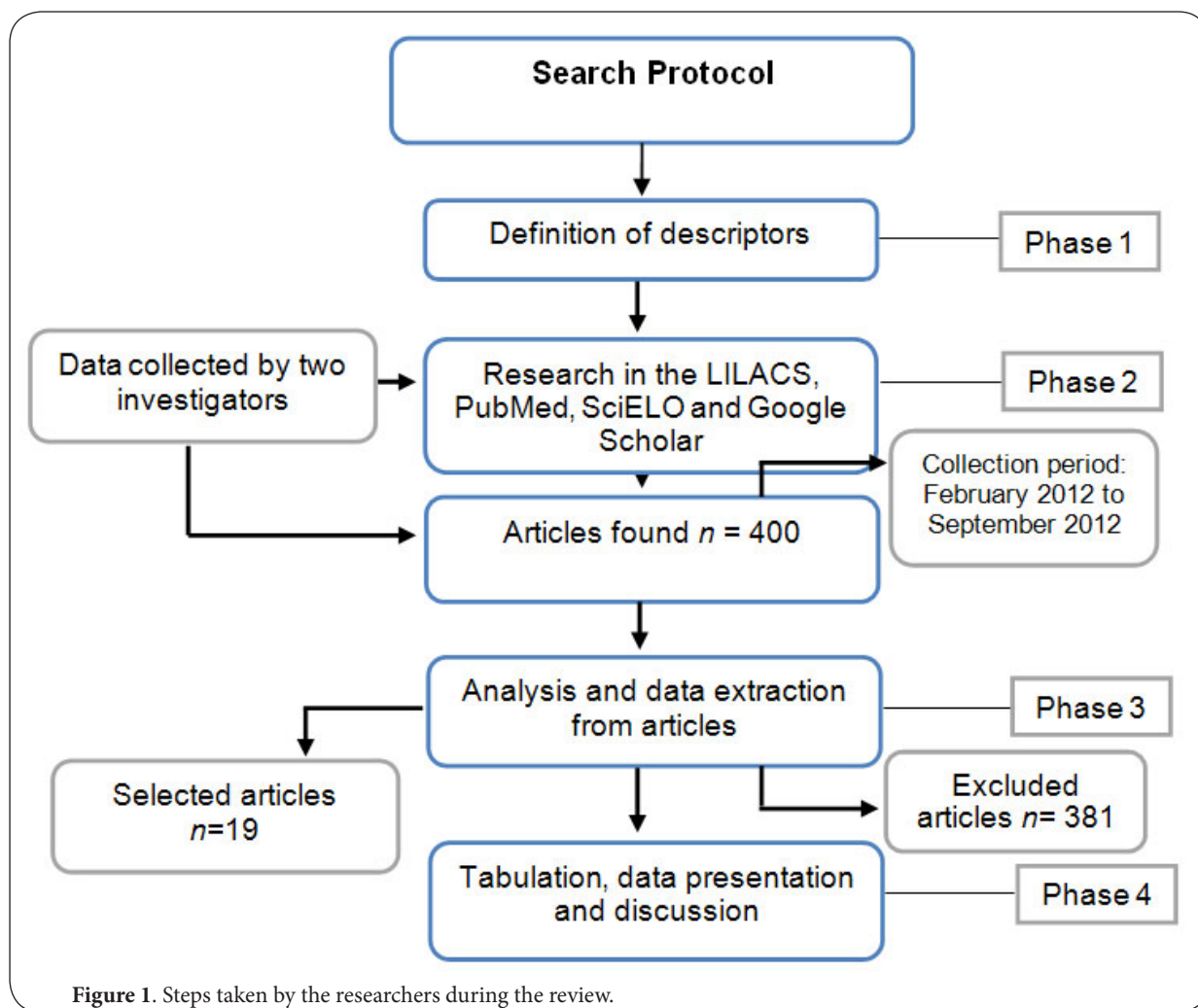


Figure 1. Steps taken by the researchers during the review.

Presentation of the results-Phase 4

The results of the studies that were included in the final analysis are presented in tables, which highlight the main characteristics of the data. Therefore, a critical summary was prepared by synthesizing the information that was extracted from the articles and by describing the variables that were studied according to the country of origin, year of publication, methodological design, clinical data, parameters and laboratory techniques.

Results

In this systematic review, articles were selected based on the use of standardized methodology with pre-defined criteria in the studies; this selection criterion was enforced uniformly. Currently, this type of review is preferred in studies because there is a greater risk of bias using other types of review [29,31].

During the completion of phases 1 and 2, a total of 400 articles were accessed from February 2012 to September 2012. After phase 3, a total of 19 articles were available for the final analysis of the study. The main reasons for the exclusion

of articles in phase 3 were a lack of detail in the techniques that were used, that only a serological diagnosis was made and that human samples were not used in the methodology. Phase 4 included the tabulation of the data from these studies. The steps that were followed during the review are outlined in a flow chart (Figure 1).

The studies that used a molecular methodology to detect viruses that were transmitted by arthropods in clinical specimens in Brazil are summarized in (Table 2). The major types of viruses that were evaluated included dengue virus (32%), OROV (22%), YFV (11%), SLEV (11%), MAYV (11%), ROCV (5%) and CPCV (5%). Most of the studies were published in 2011 (26%) and 2012 (16%). Therefore, these studies were recent, which demonstrates that a major objective of these studies was to determine the distribution of arboviruses in the Brazil using new laboratory methods.

The pioneering studies on arboviruses in Brazil were based on viruses that were isolated from the Brazilian Amazon, where the ecosystem provides conditions that are conducive for the circulation of these viruses [24]. In addition to this

Table 2. Studies in Brazil (1990-2012) that were selected for review and of other countries.

Reference	Country	Year of publication	Study design	n sample (human)	Molecular method	Arbovirus
[32]	BRAZIL	2012	Cohort study	33	RT-PCR	MAYV [†]
[33]	BRAZIL	2012	Cohort study	110	RT-PCR	OROV [‡]
[34]	BRAZIL	2012	Cohort study	2	Nested RT-PCR, Real-time RT-PCR	DENV [§]
[35]	BRAZIL	2011	Cohort study	71	Semi-nested RT-PCR, Green qRT-PCR	YFV [*]
[36]	BRAZIL	2011	Case-control	25	RT-PCR	YFV
[37]	BRAZIL	2011	Cohort study	519	RT-PCR (duplex, multiplex nested)	SLEV [¶]
[38]	BRAZIL	2011	Cohort study	94	RT-PCR	DENV
[39]	BRAZIL	2011	Case report	1	RT-PCR	CPCV ^{**}
[40]	BRAZIL	2010	Cohort study	111	RT-PCR, RT-PCR multiplex nested	DENV
[41]	BRAZIL	2010	Short report	2	Real-time RT-PCR	DENV
[42]	BRAZIL	2009	Prospective	744	RT-PCR, Real-time RT-PCR	OROV
[43]	BRAZIL	2009	Cohort study	16	Real-time RT-PCR	DENV
[44]	BRAZIL	2008	Cohort study	1	RT-PCR	ROCV ^{††}
[45]	BRAZIL	2008	Prospective	126	RT-PCR, nested RT-PCR, Real-time RT-PCR	DENV
[46]	BRAZIL	2007	Cohort study	3	RT-PCR	MAYV
[47]	BRAZIL	2007	Cohort study	234	RT-PCR	OROV
[48]	BRAZIL	2005	Cohort study	97	RT-PCR (duplex, multiplex nested)	A/ <i>I</i> ^{‡‡}
[49]	BRAZIL	2005	Case report	1	RT-PCR	SLEV
[50]	BRAZIL	2002	Cohort study	30	Nested RT-PCR	OROV
[51]	FRANCE	2012	Cohort study	69	qRT-PCR	CHIKV ^{§§}
[52]	U*	2012	Cohort study	29	Triplex RT-PCR	JEV ^{**} /GETV ^{**} /TAHV ^{***}
[53]	U	2012	Prospective	-	Multiplex RT-PCR	JEV
[54]	IRAN	2012	Prospective	100	RT-PCR	CCHFV ^{†††}
[55]	IRAN	2012	Retrospective	632	RT-PCR	WNV ^{‡‡‡}
[56]	INDIA	2012	Cohort study	513	RT-PCR	JEV
[57]	U	2011	Prospective	104	Real-Time RT-PCR	JEV
[58]	U	2007	Prospective	658	RT-PCR	CHIKV
[59]	French Guiana	2008	Retrospective	222	RT-PCR	DENV
[60]	Malaysia	2008	Cohort study	2.958	RT-PCR	DENV
[61]	Turkey	2007	Cohort study	108	RT-PCR	CCHFV

*Undisclosed; †Mayaro virus; ‡Oropouche virus; §Dengue virus; ¶Yellow fever virus; ¶Saint Louis Encephalitis virus; **Cacipacore virus; ††Rocio virus; ‡‡Alphavirus/Flavivirus; §§Chikungunya virus; **Japanese encephalitis virus; ¶¶Getah virus; ***Tahyna virus; †††Crimean-Congo hemorrhagic fever virus; ‡‡‡West Nile virus.

region, studies were conducted using viruses from the State of São Paulo, which is the most populous state in Brazil and has the largest economy in the country [62]. Additionally, samples were collected from the following cities for virological diagnosis: Oiapoque in the State of Amapá; Recife in the State of Pernambuco; Magalhães Barata, Porto de Moz, Novo Repartimento, Parauapebas, Oriximiná and Maracanã in the State of Pará; Plácido Castro in the State of Acre; Porto Velho and Theobroma in the State of Rondônia; and São Paulo, Ribeirão Preto, São José do Rio Preto, Oswaldo Cruz, Paulo Faria and Flórida Paulista in the State of São Paulo.

When the clinical signs of arbovirus infection were evaluated, the classic triad of acute fever, hemorrhagic fever and encephalitis was observed. In most cases, acute fever occurs; however, this triad can occur simultaneously, which has been observed in patients with a more severe clinical status [63]. The symptoms of the patients who were evaluated in this literature review and the laboratory parameters that were analyzed by three authors [35,37,48] are summarized in (Table 3). An overlap of the clinical status for the following infections was observed: dengue, YF, ROCV, OROV, SLEV, MAYV

and CPCV. Most of these viruses cause mild symptoms or an acute febrile phase, such as in MAYV and OROV infection. However, viruses, such as dengue [64], YF and SLEV (less frequently), may lead to death.

Discussion

In the selected studies, most researchers aimed to identify the various serotypes of dengue (DENV1, DENV2, DENV3 and DENV4) because several dengue epidemics have become constant and severe in recent years, thus representing an important public health problem. Therefore, during an epidemic of dengue-3 in the interior of the state of São Paulo, Bernardes-Terzian *et al.*, sought to identify arboviruses in biological samples from 519 individuals with acute febrile illness who were already determined to have dengue based on clinical and epidemiological data. However, when RT-PCR and the variants of this test were performed using primers that were specific for various arboviruses, DENV and SLEV were detected in only 71% and 1.5% of the samples, respectively. In 28% of the samples, arboviruses were not identified [37]. In addition, Santana *et al.*, observed a co-infection between

Table 3. Data related to the symptoms and laboratory parameters of the individuals who were assessed.

Reference	Sample collection after the onset of symptoms	Clinical samples collected	Clinical status	Laboratory parameters
[32]	> 5 days	Serum	Fever, headache, arthralgia and myalgia	NA†
[33]	U*	Cerebrospinal fluid	Meningoencephalitis	NA
[34]	U	Serum	Fever, headache and myalgia	NA
[35]	U	Serum and tissue	Similar to infection by YFV	RT-PCR: S [‡] =52.1%; Sp [§] =100%; S-N* RT-PCR: S=70.9%; Sp= 98.2%; qRT-PCR: S= 92.3%; Sp= 100%
[36]	U	Blood/serum and tissue of humans	Similar to infection by YFV	NA
[37]	≤ 5 days	Serum	Fever, headache and myalgia	RT-PCR MN [¶] : S=99%; Sp= 83% to arbovirus
[38]	≤ 5 and ≥ 3 days	Blood/serum	Clinical suspicion of dengue	NA
[39]	U	Blood/serum	Suggestive of leptospirosis and YF	NA
[40]	U	Blood/serum	Fever, headache and chills	NA
[41]	≤ 9 and ≥ 2 days	Serum, urine and saliva	Fever, headache myalgia and retroocular pain	NA
[42]	≤ 5 and ≥ 3 days	Serum	Acute febrile illness	NA
[43]	U	Blood	Compatible with symptoms of dengue	NA
[44]	≥ 5 days	Autopsied human brain specimen	Encephalitis	NA
[45]	≤ 5 days	Serum	Fever	NA
[46]	≥ 4 days	Blood/serum	High fever and arthralgia	NA
[47]	U	Blood/serum	Acute febrile illness	NA
[48]	≤ 5 days	Blood/serum	Clinical signs suggestive of arboviruses	RT-PCR D ^{**} : Sc= 64%; Sp=100%; RT-PCR MN: S= 99%; Sp= 83%
[49]	≤ 25 and ≥ 3 days	Blood/serum	Fever, headache, myalgia, eye pain, nausea, vomiting and rash	NA
[50]	U	Serum	U	NA

*undisclosed; †not assessed; ‡sensitivity; §specificity; *semi-nested; ¶multiplex nested; **duplex.

DENV and *Plasmodium sp*, which is the etiological agent of malaria [40]. Therefore, a differential diagnosis should be concurrently performed for these diseases in endemic areas.

Calzavara-Siva *et al.*, performed an experimental study to develop a diagnostic method that could differentiate between the two clinical forms of dengue (*i.e.*, classical dengue fever and hemorrhagic dengue fever). Therefore, were analyzed the expression levels of six mRNA markers of dengue hemorrhagic fever using real-time RT-PCR. In a patient who was infected with dengue, the markers could identify the evolution of the virus from the classical disease form to the hemorrhagic disease form. Despite the small sample (n = 16), these markers may be useful for the prognosis of the severe form of dengue and in a rapid assay with potential clinical use [43]. Moreover, among the studies that were included in this review, only Dos Santos *et al.*, used the ELISA to capture the NS1 antigen in the acute phase of dengue [45]. This method is more sensitive than traditional RT-PCR and is used in many laboratories for the early diagnosis of dengue [65].

In other research has been investigated Oropouche fever in epidemic areas in the Brazilian Amazon. In the study by Vasconcelos *et al.*, 64% of the samples were positive for the OROV, whereas the prevalence of the OROV among the different regions ranged from 12.7%-52.1% in the study by Azevedo *et al.*, [42,47]. Bastos *et al.*, evaluated cerebrospinal fluid samples from patients with meningoencephalitis in the state of Amazonas and detected the presence of the OROV in approximately 3% of the samples. This study demonstrated

that this disease, although rare, could be investigated in cases of meningoencephalitis with unknown etiology [33]. OROV infection is the second most common arboviral infection in Brazil with at least half a million cases [66], and many of these infections occur sporadically [67]. However, this infection has been observed in a small number of studies that were conducted in other regions of the country, excluding the Amazon region [68].

DENV epidemics have often occurred in several Brazilian municipalities, and many cases were not confirmed using laboratory methods, instead using only clinical and epidemiological data. Therefore, developing methods for the differential diagnosis of the DENV and OROV infections is important to prevent the occurrence of false positives and false negatives for dengue or Oropouche fever due to the similarity of their symptoms. Additionally, a potential vector of the OROV in urban areas is the midge *Culicoides paraensis*, which has wide geographical distribution in the Americas [67]; therefore, additional studies in different regions of Brazil should be conducted to determine the spatial distribution of this vector of the OROV.

YF has two transmission cycles: urban and wild. In the urban cycle, the infected mosquito, such as *Aedes aegypti*, which is the same vector for dengue, transmits the virus. Because of the difficulty in controlling this mosquito population and the lack of strategies for the mass vaccination of travelers, YF is considered to be a reemerging disease. In severe cases of YF, the mortality rate ranges from 20%-50% [69]. Nunes *et*

al., evaluated two new methods for the molecular detection of the YFV using the Green qRT-PCR and RT-PCR semi-nested techniques. Both techniques were useful in the early diagnosis of YFV infection and could be used in epidemiological studies of YFV [35].

Moreno *et al.*, performed an entomological study and an eco-epidemiological evaluation of 577 human samples, 108 monkey samples (*Callithrix penicillata*, *Alouatta caraya*, *Cebus apella* and other species) and 3 049 midge samples that were collected in the State of São Paulo. Viral isolation, ELISA-IgM, RT-PCR, histopathology and immunohistochemistry confirmed the sporadic circulation of the YFV [36]. Deubel *et al.*, isolated the YFV from a fatal case of YF that involved an individual who returned to France after a trip in endemic areas of the Amazon region and who was not vaccinated [69]. Therefore, vaccination against YF in areas that are at risk for travelers and for the populations in these locations is important.

In Brazil, several serological studies have been conducted to elucidate the SLEV [37,70]. Antibodies against arbovirus have been observed in 10% of horses and in 5% of the human population in the North and Northeast regions [70]. However, the hemagglutination inhibition and ELISA serological tests have demonstrated that cross-reactions between antibodies from different *Flaviviruses* occur; these reactions are specifically induced by exposure to DENV and in individuals who are vaccinated against YF. In this context, include the ROCV and MAYV, which have caused outbreaks in Manaus, may result in cross-reactivity between several arboviruses [32,46].

Our search for articles in the international scientific literature demonstrated that studies on the differential diagnosis of arboviruses are rare. In addition, a pilot study that originated from countries in South America (*i.e.*, Bolivia, Ecuador, Paraguay and Peru) has garnered attention [71]. This multicenter study was conducted from 2000-2007, and the researchers collected 20 880 samples from patients with febrile illness in the acute or convalescent phases using immunofluorescence, RT-PCR and ELISA-IgM. Co-infection or a recent infection with arboviruses was detected in 32.5% of the samples, among which 26% were infected with the DENV and 3% were infected with the VEEV, MAYV, OROV, group C virus or Guaroa virus. However, 63.5% of the samples were collected from patients in the convalescent phase, which may have decreased the sensitivity of the technique.

In this systematic review, we concluded that there are no standardized molecular techniques for the diagnosis of arboviruses, and most of the molecular methods were used *in house*. However, several authors have focused on developing new tests. Additionally, cross-reactivity and the immune window period are drawbacks in the use of serological methods. However, many patients who had an infection that was suggestive of arbovirus infection were not diagnosed, even when accurate techniques, such as RT-PCR, were used. Therefore, it remains unclear whether these tests resulted in a false negative or whether other etiological agents were present.

A diagnosis that is based only on clinical and epidemiological data would be premature. Therefore, new entomological research and new differential molecular methods should be performed for the possible isolation of these unknown viruses to contribute to the diagnosis of arboviruses.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MLM and VGC developed and wrote the study protocol. MLM and VGC conducted a search of the scientific literature and analyzed articles according to the selection criteria. MLM and VGC evaluated the data from the studies that were included in the systematic review and wrote the manuscript. Both authors read and approved the final version of the manuscript.

Publication history

Received: 22-Feb-2013 Revised: 24-Mar-2013

Accepted: 26-Mar-2013 Published: 29-Mar-2013

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Citation:

Moreli M L and da Costa V G: **A systematic review of molecular diagnostic methods for the detection of arboviruses in clinical specimens in Brazil and the importance of a differential diagnosis.** *Virology Discovery* 2013, **1**:1.
<http://dx.doi.org/10.7243/2052-6202-1-1>