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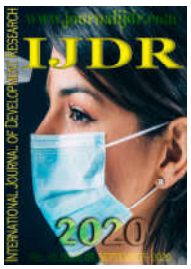

RESEARCH ARTICLE OPEN ACCESS

DETECTION ALL FOUR SEROTYPES OF DENGUE VIRUS IN *AEDES AEGYPTI* MOSQUITOES CAPTURED IN TRES LAGOAS – MS, BRAZIL

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ABSTRACT

Três Lagoas - MS has favorable characteristics for the reproduction and maintenance of the Dengue vírus (DENV) vector ranked 3th in the number of cases and inicdence. This study aimed to capture of *Aedes aegypti* to verify the circulation of DENV serotypes in this city. Entomological surveys were carried in urban area in nine collection points, covering of the city and using Adultrap® traps. After capture all the mosquitoes were identification, and the females were used to detect the DENV with later serotyping by RT-*Nested*-PCR. Larvae also were captured in many places of city and analyzed by similar technique. A total of 18,541 adults mosquitoes were captured, where 6,856 (36.98%) were identified as *A, aegypti*, with an average female density of 5.74 and prevalence of DENV1, DENV2, DENV4 and DENV3 respectivaley. A total of 1,173 larvae were collected, of which 348 were of the species *A. aegypti* (49.5%). The DENV detection showed seven positive pools (53.84%) with prevalence of similar serotypes found in adults mosquitoes. Tres Lagoas, have a high infestation of *A. aegypti* with high DENV positivity with circulation of all four DENV serotypes, corroborating the high number of human dengue cases occurs anually.

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INTRODUCTION

Arboviruses are diseases transmitted by an ecologically well-defined group of viruses which derives from the expression Arthropod-Borne Viruses, (Gubler 2002, Figueiredo *et al*., 2007). Among arboviruses, Dengue (DENV) is the most important disease transmitted by mosquitoes worldwide, and responsible for a serious public health problem in Brazil and in other countries in the world (Brazil, 2019a). The dengue virus group has four distinct serotypes: DENV1, DENV2, DENV3 and DENV4, which are antigenically and phylogenetically distinct. These serotypes have the same transmission mechanism and cause human disease with similar clinical manifestations (Pierson *et al*., 2012, Mackenzie *et al*., 2004). The maintenance theses virus occurs by an urban transmission cycle: "man - mosquito - man", where the mosquitoes *Aedes aegypti* and *Aedes albopictus* are the main vectors and due to its urban habits, the *A. aegypti* is the most important vector (Marcondes 2015, Kraemer 2015). The arthropod is the real reservoir of arboviruses remains infected and infectious for the rest of its life, becoming unable to control the invasion of the virus in all parts of its body, including the gonads, allowing vertical transmission (Guzman, 2010). The vector density is

directly related to the number of cases of the disease, and to climatic factors that favor its reproduction. However, the virus can maintain a considerable population during the less rainy seasons, at the expense of semi-permanent breeding sites independent of the rains. Their preferred breeding sites are artificial containers, both those abandoned by man in the open and filled with rainwater, as well as those used to store water for domestic use. In addition, they can produce eggs resistant to desiccation and remain viable for up to a year (Consoli, 1994). Brazil has geographical, climatic and ecological conditions for the maintenance and breeding of mosquitoes, making dengue a serious public health problem. Epidemiological analyzes, between 2010 and 2019, show more than 11 million news cases, including more than 5,000 deaths by severe dengue (Stolerman *et al*., 2019, Brasil, 2019a). In the state of Mato Grosso do Sul (MS) have been notified more than 60,000 cases of Dengue in 2019, with 35,894 confirmed cases. This index is well above of previous years: 10,729 cases in 2018 and 7,276 cases in 2017, but similar to 2016, with 65,505 notified cases, estimating a high incidence of 2,575 (Brasil, 2019b). Três Lagoas - MS, during 2019, ranked 3th in the number of cases in the state, having 5,732 notified cases and an incidence of 2,228/100,000 inhabitants, similar to found in 2018 when 3,086 cases were reported with an estimated incidence of 2,814, ranked first in the number of cases in the state. These high incidences can be associated with geographic, climatic and ecological characteristics appropriate to the reproduction and maintenance of the vector (Três Lagoas, 2020). In this context, this study aimed to capture *A. aegypti* (larvae and adults) to verify the presence of DENV serotypes in mosquitoes in the Três Lagoas city.

MATERIALS AND METHODS

Study area: Três Lagoas is the third largest city in the state of Mato Grosso do Sul (Figure 1). It is located in the eastern region of the state, 337 km from the capital and 320 m above sea level (20º45'35 "south latitude and 51º41'42" longitude west), with a total area of 10,206 km^2 and an approximate population of 121,388 inhabitants (IBGE, 2019).

Mosquito and larvae collection: Entomological surveys were carried in urban area of Três Lagoas from September to November 2019. Nine collection points, covering of the city, were selected and numbered from 1 to 9. The central point of each area was geolocalized and from this point, three concentric circular areas (100, 350 and 700 meters from the central point) were calculated, placing adult insect capture traps on the edge of these areas. Were used 15 Adultrap® traps: four traps in the first circle (100 m), five traps in the second circle (350 m) and six traps in the third circle (750 m). The traps were allocated in peridomiciliar regions (balconies, backyards, open garages, among others) and/or vacant lots, remaining in these locations for 5 days. Daily, the mosquitoes captured were collected and pooled by areas and stored on dry ice before transportation to the Virology and Cell Culture laboratory of the Universidade Federal de Mato Grosso do Sul (UFMS), where they were stored at -80ºC until further processing. Larvae samples were collected in different regions of the city, at all maturation stage (L1, L2, L3, L4 and pupa). The larvae collection was performed September to November 2019 and after capture were sent on the same day to Laboratory of Virology and Cell Culture of the UFMS. All the capture larvae were placed separately in plastic cups containing 10 mL of water, for complete metamorphosis and obtaining of mosquitoes. The larvae were maintained at a temperature of 27ºC, fed with crushed cat food and their metamorphosis was monitored daily. After mosquitoes obtaining they were collected and stored at -80ºC until further processing. Mosquitoes were identified on a frozen table using morphological identification keys (Rueda 2004) separating in *A. aegypti*, *Culex* sp. and others. Mosquitoes identified as *A. aegypti* were separated by sex, counted and pooled in 15 females (1 sample) from each area where sufficient mosquitoes were collected. The mosquitoes obtained by larvae capture, were pooled randomly. The male mosquitoes of *A. aegypti* and mosquitoes of others species were again stored at -80ºC for further investigation. The average density of females in an area was determined by the formula: number of females caught/(quantities of traps on site x days of capture (5 days)).

RNA extraction: Mosquito pools containing 15 specimens/pool were macerated using a crucible and pistil in 600 µL of Dulbecco's modified Eagle's medium culture medium with foetal bovine serum and antibiotics (penicillin-streptomycin). Samples were centrifuged and the global aliquot (600 µL) was separated into 2 aliquots (300 µL each), one used for viral RNA extraction, using the Qiamp Viral RNA Kit (Qiagen Inc., USA) and another one stored at -80ºC for future studies. The aliquots of viral RNA were stored at -80°C until further use.

RT-PCR and Multiplex Nested – PCR: DENV were detected using the extracted RNA following the protocol described by Bronzoni *et al*. (2005). In summary, the RNA was subjected to Reverse Transcriptase using FG2R-GTGTCCCATCCTGCTGTGTCATCAGCATACA. The amplification product was used in polymerase chain reaction (PCR) adding FG1 F-TCAAGGAACT CCACACAT GAGATGTACT. Both primers are flavivirus gender-specific and amplify a fragment of 1000 base pairs (bp). The PCR product was diluted 10-fold and newly amplified using FG1F primer and specific primers for DENV serotype: DENV1R-CGTTTTGCTCTTGTGTGCGC (Amplification product: 472 bp), DENV2R- GAACCAGTTTG GTT DRTTTCAT CGCTGCC (Amplification product: 316 bp), DENV3R-TTCCTCGTC CTCAA CAGCAGCTCTCGCACT (Amplification product: 659 bp) and DENV4R-GCAATCGCTGAAGCCTTCTCCC (Amplification product: 222 bp). Whenever Flavivirus gender was detected, the multiplex nested PCR, using specifics primers to each serotypes, was performed. The amplification products were confirmed by electrophoresis agarose gel, ethidium bromide-stained and visualized on UV. Positive controls were obtain by viral RNA extraction of supernatants of C6/36 mosquito cells infected with each DENV serotypes. Supernatant of C6/36 mosquito cells of uninfected

RESULTS

A total of 18,541 adults mosquitoes were captured, where 6,856 (36.98%) were identified as *Aedes aegypti*, 9,359 (50.48%) identified as *Culex sp.* and 2,326 (12.54%) pooled as others. Among the 6,856 identified as *Aedes aegypti*, 2,980 (43.47%) were male and 3,876 (56.53%) were females, with an average female density of 5.74 with large variations from 2.9 to 11.6. (Table 1). Three points showed high level of mosquitoes infestation with a increase in the average females density, which highlight: Point 3, with 1,107 mosquitoes, 635 females (55.6%) and average female density of 8.68, Point 7,

Figure 1. Location of the municipality of Três Lagoas – MS

Table 1. Capture points, classification of captured mosquitoes and detection of Dengue / serotypes.

			Aedes aegypti			Analyzed					
Points	Geographic location	Radius (m)	Male	Female	Female Density	Pools	DENV positive	D1	D2	D3	D4
1	20°46'20.5" S	A1 (100 m)	96	115	5,75	7	3	X	X	-	-
	51°41'15.2"	B1 (350 m)	122	174	6,96	11	5	X	X	-	X
	W	C1 (700 m)	91	98	3,26	6	3	X	-	X	X
2	20°46'12.6" S	A2 (100 m)	125	118	5,9	7	4	X	-	-	-
	51°41'58.7"	B2 (350 m)	83	132	5,28	8	6	X	X	-	X
	W	C2 (700 m)	101	204	6,8	13	8	X	-	-	X
3	20°46'47" S	A3 (100 m)	139	232	11,6	15	10	X	X	-	-
	51°42'41.5"	B3 (350 m)	144	154	6,16	10	7	X	X	-	X
	W	C3 (700 m)	189	249	8,3	16	11	X	X	-	X
4	20°47'39" S	A4 (100 m)	151	136	6,8	9	4	X	X	-	X
	51°43'08.4"	B4 (350 m)	90	93	3,72	6	4	-	X	-	-
	W	C4 (700 m)	38	81	3,24	5	1	X	-	-	-
5	20°48'14.4" S	A5 (100 m)	64	101	3,36	6	3	X	-	-	-
	51°42'51.7"	B5 (350 m)	93	116	4,64	7	5	X	X	-	-
	W	C5 (700 m)	189	167	5,56	11	7	X	-	-	X
6	20°48'10.9" S	A6 (100 m)	127	182	9,01	12	6	X	X	-	X
	51°41'51.9"	B6 (350 m)	49	96	3,84	6	4	X	-	-	-
	W	C6 (700 m)	113	190	6,33	12	7	X	X	-	-
7	20°46'59.3" S	A7 (100 m)	159	169	8,45	11	7	X	X	-	-
	51°41'40.4"	B7 (350 m)	146	251	10,04	16	9	X	X	-	-
	W	C7 (700 m)	96	144	4,8	9	5	X	-	X	X
8	20°46'42.7" S	A8 (100 m)	103	121	6,05	8	5	X	X	-	X
	51°40'42.5"	B8 (350 m)	67	135	5,4	9	6	X	X	-	X
	W	C8 (700 m)	128	127	4,23	8	4	-	X	X	X
9	20°47'25.9" S	A9 (100 m)	97	93	4,65	6	3	X	-	-	-
	51°39'48.2"	B9 (350 m)	91	111	4,44	7	4	X	X	-	X
	W	C9 (700 m)	89	87	2,9	5	1	X	-	X	-
Total			2980	3876	5,74	246	142	25	17	04	14

with 965 mosquitoes, 564 females (58.4%) and average female density of 7.76 and Point 6, with 757 mosquitoes, 468 females (61.8%) and an average female density of 6.39. One point showed low level of mosquitoes infestation with decrease in the average female density: Point 9, with 568 mosquitoes, 291 females (51.2%) and average female density of 9.7 (Table 1). A total of 246 pools were analyzed, where 142 pools were considered positive for one or more DENV serotypes (57.72%). Four points showed a high percentage of positivity (positive ratio x number of analyzed pools): Point 3 with 68.3% of positive pools, Point 5 with 62.5% of positive pools, 8 with 60% positive pools and 7 with 58.33% positive pools. Two points showed a lower positivity: Point 9 with 44.4% of positive pools, Point 4 with 45% of positive pools and Point 1 with 45.83% of positive pools. The serotype detection showed a predominance in the circulation of DENV1 followed by DENV2, which were detected in all analyzed points. The serotype DENV4 serotype was also found in all analyzed points, but less frequently, when compared to the presence of DENV 1 and DENV2. The DENV3 serotype was the least frequent serotype, detected only at points 1, 7, 8 and 9 (Table 3). A total of 1,173 larvae were collected, in different stages of differentiation (L1, L2, L3, L4 and pulp), allowing to obtain 703 adult mosquitoes, of which 348 mosquitoes were of the species *Aedes aegypti* (49.5%) and 355 were of other species, with a prevalence of mosquitoes of the genus *Culex spp* (51.55%). Among the mosquitoes of the species *A. aegypti*, 197 were females (56.61%) and 151 males (43.39%), allowing to obtain 13 pools (15 mosquitoes/pool). The DENV detection showed sevem positive pools (53.84%) a positivity value similar to found in the detection of captured adult mosquitoes. The serotype detection showed a predominance circulation of DENV1 and DENV2, which were detected in 5 samples. The serotype DENV4 and DEN3 were detected only one sample each.

DISCUSSION

This study showed that *A. aegypti* is abundant in all urban perimeter of Três Lagoas city, with detection of all four DENV serotypes within a study area. DENV infected mosquitoes were detected in approximately 58% of the mosquito pools and in all points where mosquitoes were collected. We detected high mosquito densities with an average of up to five *A. aegypti* female per point/day. Similar to that detected in adult mosquitoes captured, the detection of DENV in larvae samples also showed high rates of positivity (53.84% - 7 pools of 13 total pools). Similar studies with larvae samples performed in Manaus - AM, analyzed larvae samples captured in residences of DENV patients showed an average infection of 53%, with variations between neighborhoods ranging from 30 to 70% (Costa *et al*., 2009). Study carried out in Ciudad de Juarez, Chihuahua, México, obseved 57% of captured mosquitoes were infected, with variation within municipality ranging between 47 a 60% (Mora-Covarrubias *et al*., 2010). On the other hand, similar study performed in Recife - PE showed infection rates of 12%, much lower than that found in our analyzes (Guedes *et al*., 2010). The Breteau Index (BI) assessed by the municipality's entomology sector and which measures the level of *A. aegypti* infestation, was compared to our analyzes (female density), showing a strong correlation between both findings in the same period. Therefore, all points with high density detection of *A. aegypti* females by this study, showed high rates of BI. In addition, the points detected with a high rate of infestation also showed a high rate of human DENV cases (358 suspected cases), as reported by the municipality's epidemiological surveillance department (Três Lagoas, 2020).

In a study performed by Sanchez *et al*., (2006) and Thammapalo *et al*., (2008) were evaluated the possibility of predicting dengue transmission using BI, where one block of houses with BI ≥4 in a neighbourhood predicted DENV transmission with a sensitivity of 78% and a specificity of 63%. Our results corroborate these findings by showing that the female density indexes correlate to BI and also to the number of human DENV cases in an area. A quantitative comparison with other studies is difficult, due to different collection methods, however our studies are corroborated for analysis performed by Mondini *et al*., (2005), San Pedro *et al*., (2009), Almeida *et al*., (2007), Barcellos *et al*., (2005) and Ribeiro *et al*., (2006), among others, who report a significant correlation between mosquitoes infestation and human cases, in different municipalities of Brazil. Studies carried out in Peru by Getis *et al*., (2003) and Chowell *et al*., (2008) also demonstrate a strong correlation between infestation and human cases. An important highlight point is the catches of adult mosquitoes were performed between September to November 2019, where there was moderate rain and high temperatures, which are characteristics that contribute to the reproduction of the mosquitoes, generating high degrees of infestation. Many factors support proliferation of *A. aegypti* and consequently the sustained transmission of mosquito-borne diseases in Brazil, including the climate, thus the rainfall affects mosquito abundance positively through the creation of new breeding sites, though the vector is able to adapt to new environmental situations (Simões *et al*., 2013, Teixeira *et al*., 2009, Gómez-Dantés *et al*., 2009).

In Brazil, all four DENV serotypes have been in cirulation since 2010 with circulation of one or more serotypes in different regions and causing several outbreaks in all Brazilian states (Romano *et al*., 2010). After determining of the positivity DENV mosquitoes, it was possible to analyze which virus serotypes circulate in the municipality. We observed a predominance in the circulation of DENV1 serotype viruses, followed by DENV2, which were detected in all analyzed points. The DENV4 serotype was also found in all analyzed points, but less frequently, when compared to the presence of DENV 1 and DENV2. The DENV3 serotype was the least frequent serotype, being detected only at points 1, 7, 8 and 9. Among the serotypes found in larvae samples we also observed a predominance of DENV1 and DENV2, found in five samples. The DENV4 and DENV3 serotype were found in only one samples each. Due to the fact that the larvae samples were not separated by the place of capture, it was not possible to establish whether the region of origin of the larvae pool with DENV3 serotype detection corresponded to the region of DENV3 detection in adult mosquitoes. Fares *et al*., (2015), studing the epidemiological scenario of dengue in Brazil, showed cirlutaion of DENV1, DENV2 and DENV4 in the Mato Grosso do Sul state. However, in the same article can be observed the circulation of DENV3 in the Minas Gerais state. In the context it is important to highlight that Três Lagoas, is the gateway to the state, receiving people and cargo daily from its neighboring states (Minas Gerais, São Paulo, Goias) facilitating the insertion of a new virus serotype (Figure 1). Corroborating this fact, a study performed by Zanotto *et al*. (2018) showed that the São Paulo state have the circulation of all four DENV serotypes. In 2012 was registered the insertion

of DENV4 in Mato Grosso do Sul state, which was associated to its presence in bordering states and countries, combined with high levels of infestation by *A. aegypti* and a susceptible population resulted in the massive epidemic (Bertolacci-Rocha *et al*., 2014). However, despite the detection of DENV3 insertion in the municipality (infected mosquitoes), human cases of DENV3 infection has not been reported so far (Três Lagoas, 2019). The infection by more one DENV serotype in the similar mosquito was described by Thavara *et al*., (2006) in the southern Thailand. We detected infection by two serotypes in five mosquitoes pools, however we believe that these reflect single infections in separate mosquitoes. Pérez-Castro *et al*., (2016) analyzed DENV serotypes circulation in female mosquitoes collected in a rural area in Colombia also detected multiple serotypes in same pools, attributng this to an infection in separate mosquitoes. It is worth noting that there was no detection of multiple serotypes in the larvae samples, which can be explained by the smaller number of analyzed pool, since the infection of multiple serotype in larvae has also been demonstrated (Bara *et al*., 2013). Other important point of the detection of DENV in larvae samples is the corroboration to the evidence of vertical transmission, since the larvae were transformed in adult on the laboratory, not having access to blood. Thus, this results reinforcing numerous studies that demonstrate the capacity of DENV vertical transmission as an important factor for infection maintenance in the vector (Guzmán *et al*., 2010, Le Goff *et al*., 2011). Although the analyses was done only in female mosquitoes, males mosquitoes can also be infected with DENV, can transmit to females and act as a reservoir of viruses during interepidemics periods (Bara *et al*., 2013). Continuous assessment of DENV circulation is a important tool for predicting outbreaks and understanding the disease dynamic, allowed controls and prevention measures. Thus our results suggest the need of efficient measures to control the *A. aegypti* in the municpality, since was detected a high degree of mosquitoes infestation and the circulation of all four DENV serotypes. Yet, we emphasize that due to the geographic characteristics of the municipality, it can become a virus disperser/receptor, mainly to regions of São Paulo and Minas Gerais state.

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