

Frequency of histopathological changes in Howler monkeys (*Alouatta* sp.) naturally infected with yellow fever virus in Brazil

Silvana Gomes Leal^[1], Alessandro Pecego Martins Romano^[1], Rafael Veríssimo Monteiro^[2], Cristiano Barros de Melo^[2], Pedro Fernando da Costa Vasconcelos^[3] and Márcio Botelho de Castro^[2]

[1]. Departamento de Vigilância das Doenças Transmissíveis, Secretaria de Vigilância em Saúde, Ministério da Saúde, Brasília, Distrito Federal, Brasil. [2]. Faculdade de Agronomia e Veterinária, Universidade de Brasília, Brasília, Distrito Federal, Brasil. [3]. Instituto Evandro Chagas, Secretaria de Vigilância em Saúde, Ministério da Saúde, Belém, Pará, Brasil.

ABSTRACT

Introduction: Due to the importance that Howler monkeys have on the yellow fever (YF) epidemiological sylvatic cycle in Brazil, more accurate morphological diagnostic criteria needs to be established, especially considering the differences that may exist between the genera of Brazilian non-human primates (NHPs) involved in yellow fever virus (YFV) epizootics. **Methods:** Records of YF epizootics in NHPs in Brazil between 2007 and 2009 were obtained from the Brazilian Ministry of Health database to select YF positive (n=98) Howler monkeys (*Alouatta* sp.) for this study. The changes described in the histopathological reports were categorized by organ and their frequencies calculated. **Results:** The most frequent lesions observed in the animals with YF were hepatocyte apoptosis (Councilman body formation), midzonal hepatocyte necrosis, steatosis, liver hemorrhage, inflammatory mononuclear cell infiltration of the liver, renal acute tubular necrosis and interstitial nephritis. Midzonal hepatocyte necrosis, steatosis and hemorrhage presented positive correlations with apoptosis of hepatocytes, suggesting strong YFV pathogenic effect association; they were also the main histopathological changes in the *Alouatta* sp. A pronounced negative correlation between apoptosis of hepatocytes and hepatic mononuclear cell infiltration pointed to significant histopathological differences between YFV infection in Howler monkeys and humans. **Conclusions:** The results warn that NHPs may exhibit different response patterns following YFV infection and require a more careful diagnosis. Presumptive diagnosis based on primate histopathological lesions may contribute to public health service control.

Keywords: Yellow fever. *Alouatta*. Public health. Diagnostic.

INTRODUCTION

Yellow fever (YF) is an acute febrile infectious disease with a high mortality rate, caused by a flavivirus and transmitted to humans by bloodsucking mosquitoes. In Brazil, YF occurs almost entirely in the wild environment, where non-human primates (NHPs) are the primary susceptible hosts, playing a central role in the disease's epidemiology^{(1) (2) (3) (4)}.

The morphologic diagnosis of YF remains one of the most important tools in the identification of the disease, due to the typical morphological characteristics of the lesions associated with viral infection confirmation methods. Histopathological diagnosis is based on characteristic injury patterns such as midzonal necrosis and liver steatosis, the presence of Councilman bodies, and the paucity of hepatic inflammation and hemorrhage^{(5) (6) (7) (8)}.

Experimental animal models have allowed characterization of the clinical and pathological changes and pathogenesis of yellow fever virus (YFV) infection^{(9) (10)}. These animal models led to the validation of YF histopathological diagnosis patterns that are observed in almost all human cases^{(4) (5) (8)}.

However, there are few descriptions of YF histopathological lesions in NHPs during epizootics throughout the world. The different primate species can range widely in their susceptibility to YFV infection. Indeed, rhesus and Howler monkeys are considered to be more susceptible to YFV, while marmosets and capuchin monkeys present variable degrees of resistance, and scarcely develop hepatic lesions^{(11) (12)}.

Determination of the frequency of the main histopathological changes in NHPs infected during YF epizootics in Brazil is of great importance due to its continental dimension and the ideal conditions for the spread of YFV among NHPs in the endemic wild environment. Therefore this study can contribute to improving the disease diagnostic criteria, expand monitoring strategies at the interface between human and animal health, and increase knowledge of the disease in NHPs in the natural environment.

Corresponding author: Dr. Márcio Botelho de Castro.

e-mail: mbcastro@unb.br

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METHODS

Records of YF epizootics in NHPs were determined from the Eco-epidemiological Surveillance Service database of the Brazilian Ministry of Health. The records contained a total of 2,272 epizootics involving 4,339 NHPs from 2007 to 2009. Of this total, 474 (10.9%) monkeys were confirmed with YFV infection in 232 epizootics.

Among the positive animals, Howler monkeys (*Alouatta* sp.) were selected (n=98) for this study first due to their major importance in YF epidemiology in Brazil, and second because we were able to obtain complete records and detailed descriptions of the pathological changes in organ and tissue samples. All tissue samples were fixed in 10% buffered formalin, paraffin embedded processed, and 5µm sections were stained with hematoxylin and eosin (H&E) for histopathological diagnosis. Definitive diagnosis of YFV infection was conducted at the National Reference Laboratory, Evandro Chagas Institute, Ananindeua, Pará, and the Regional Laboratory, Adolfo Lutz Institute, São Paulo, using the immunoperoxidase staining method. This involved incubating liver samples from Howler monkeys overnight with anti-YF primary antibody that was produced and standardized at the Evandro Chagas Institute.

The main information analyzed from the YF epizootic report forms included the city, federal unit and the sort of environment where the primate was found dead. Descriptive information of lesions in the liver, kidney, spleen, heart, lung and brain of YF-infected NHPs contained in the reference laboratory histopathological reports were recorded. The changes described in the histopathological reports were categorized by organ and their frequencies calculated.

Fisher's exact test with a significance level set at 0.05 was performed to test the correlation between pairs of the most frequent histological changes ($\geq 20\%$). The alternative hypotheses considered were: a) positive association between histological lesions, which occurred concomitantly; and b) change absent in the presence of the other. All statistical analyses were performed with R Statistical Software version 3.2.0, open source free software.

RESULTS

During the evaluation period, 4,339 animals died during epizootics; Howler monkeys were the most severely affected, accounting for 64.8% of deaths. Despite YF cases in other NHPs species occurring in Brazil, Howler monkeys accounted for 59.7% (1,317 of 2,272) of all YF epizootics notifications in the Ministry of Health database from 2007 to 2009.

Diagnosis of YFV infection was confirmed in only 474 NHPs and Howler monkeys were involved in 92.8% (n=440) of YF deaths. Marmosets (*Callithrix* sp. n=20), capuchin monkeys (*Sapajus* sp. n=10), and unidentified species of primate (n=4) accounted for only 7.2% of YF cases recognized in the period and were not included in this study.

Howler monkeys were found dead predominantly in rural environments (87.9%), with 5% found in urban areas during YF epizootics. However, this information was not obtained in 7.1% of the cases. During the study period, 86.7% of YF primate infections occurred in the State of Rio Grande do Sul, distributed among 64 different municipalities. The States of Minas Gerais and Goiás each accounted for 4.1% of cases, distributed among four different counties in each state. The State of São Paulo contributed 4.1% of YF cases distributed among three municipalities, and 1% of cases were in the Federal District (**Figure 1**).

The main histopathological changes and their frequencies observed in YF-infected NHPs in Brazil are presented in **Table 1**. The liver (**Figure 2** and **Figure 3**) and kidneys showed the most significant changes and highest frequency of injuries.

Midzonal necrosis was more frequent among cases with apoptosis of hepatocytes compared with cases without apoptosis of hepatocytes (87.8% versus 12.2%, $p = 0.037$). Frequency of monkeys with hepatic steatosis and apoptosis of hepatocytes were higher than samples with hepatocytes without apoptosis (94.3% versus 5.8%, $p < 0.001$) Positive association was also demonstrated between hemorrhage and apoptosis of hepatocytes when compared with samples without hepatocytes apoptosis (90% versus 10%, $p = 0.024$).

Another important change was the strong negative correlation between liver inflammatory mononuclear cell infiltration and hepatocytes with apoptosis when compared with samples without hepatocyte apoptosis (18.1% versus 81.9%, $p < 0.001$).

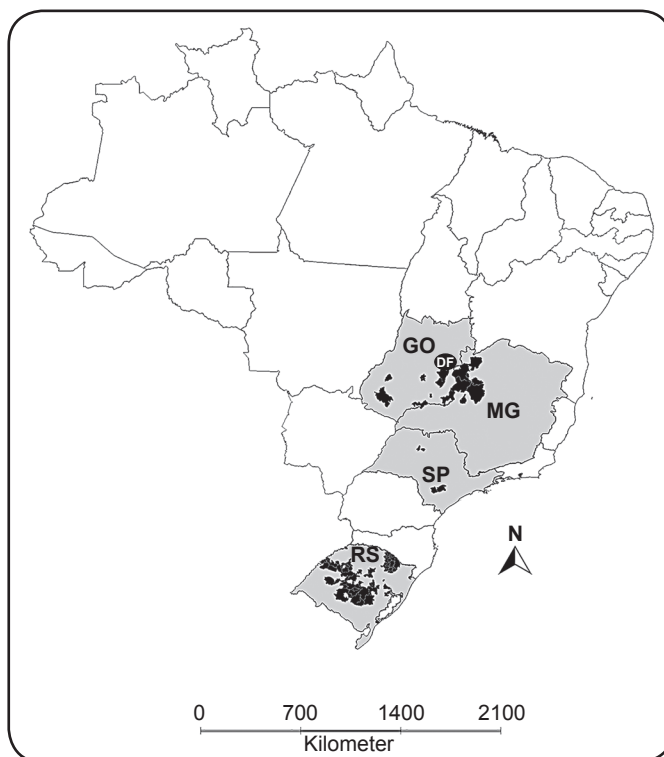


FIGURE 1 - Yellow fever outbreaks in Howler monkeys in Brazil, 2007-2009. GO: State of Goiás; DF: Distrito Federal; MG: State of Minas Gerais; SP: State of São Paulo; RS: State of Rio Grande do Sul.

TABLE 1 - Frequency of main histopathological changes in Howler monkeys naturally infected with yellow fever virus in Brazil during 2007 to 2009.

Organ	Histopathological changes	Number	Percentage
Liver (n=98)	hepatocyte apoptosis (Councilman bodies)	73	74.5
	midzonal necrosis	40	40.8
	hepatocyte steatosis	39	39.8
	parenchymal hemorrhage	30	30.6
	inflammatory mononuclear cells infiltration	19	19.4
	autolysis	19	19.4
Kidneys (n=71)	acute tubular necrosis	42	59.1
	interstitial nephritis	24	33.8
	glomerulitis	2	2.8
	autolysis	21	29.5
	no changes	4	5.5
Spleen (n=66)	lymphoid hiperplasia	6	9.1
	red pulp hyperplasia	19	28.8
	hemorrhagic	17	25.7
	congestion	17	25.7
	autolysis	24	36.3
	no changes	3	4.5
Heart (n=70)	hemorrhage	1	1.5
	congestion	5	7.1
	edema	2	2.8
	autolysis	6	8.5
	no changes	60	85.7
Lungs (n=61)	pneumonia interstitial	11	18.0
	alveolar edema	7	11.5
	hemorrhage	8	13.1
	congestion	7	11.5
	autolysis	6	9.8
	no changes	32	52.4

DISCUSSION

Despite YF being known as a disease for centuries, some aspects of its pathogenesis in primates remain to be determined. NHPs play an important role in YF epidemiological surveillance to identify sentinel events, allowing the establishment of control and prevention measures⁽¹³⁾.

Nearly all the NHPs infected with YF during the study period were recorded in rural areas of Rio Grande do Sul in 2008, a state that experienced an epizootic of great magnitude⁽¹⁴⁾. The disease has been described in Brazil only in its sylvatic cycle⁽³⁾⁽¹³⁾. The displacement of YF-positive NHPs between wild, peri-urban

and urban environments should be considered with caution, to avoid erroneous association of YFV infection with urban areas.

During the study period, Howler monkeys were involved in most YF epizootics in Brazil, possibly because they are one of the most abundant species in the natural environment and inhabit a more favorable environmental stratum for acquiring the disease⁽¹³⁾⁽¹⁴⁾. In addition, Howler monkeys are considered highly susceptible to YFV⁽¹⁵⁾, while other genera such as *Cebus* sp. and *Callithrix* sp. may exhibit variable degrees of disease resistance⁽¹¹⁾⁽¹²⁾. The distribution of Brazilian NHP epizootics, despite the great diversity of primate species in the different Brazilian biomes, strengthens the importance of Howler monkeys in YF epidemiology and their sensitivity to YFV.

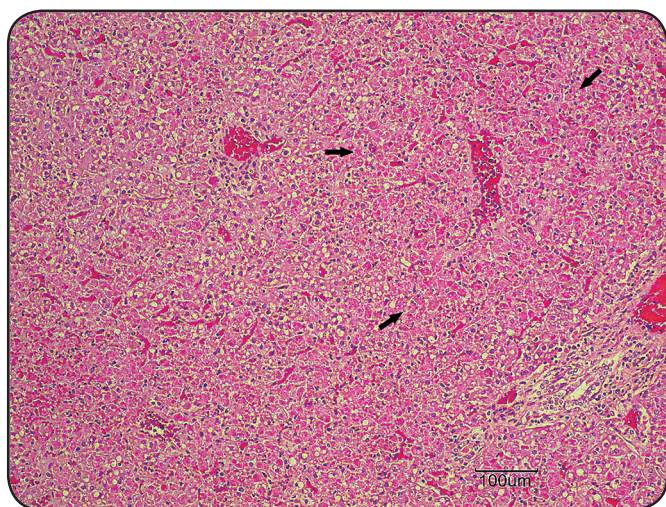


FIGURE 2 - Liver from a Howler monkey naturally infected with YFV demonstrating midzonal hepatocyte necrosis (arrows), steatosis and hemorrhage. H&E, 100X. YFV: yellow fever virus; H&E: hematoxylin and eosin.

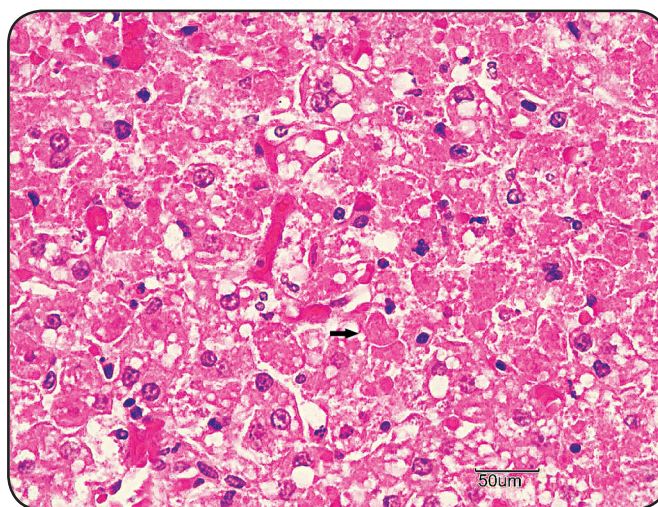


FIGURE 3 - Councilman bodies (arrow) in the liver of a Howler monkey with acute YFV infection. H&E, 400 x. YFV: yellow fever virus; H&E: hematoxylin and eosin.

The most frequent liver changes observed among YFV-infected primates in Brazil were observed in natural and experimental YFV infections^{(2) (10) (16)}. Midzonal hepatocyte necrosis, steatosis and hemorrhage showed a positive correlation with apoptosis of hepatocytes, suggesting its association with a strong YFV pathogenic effect. These results also highlight the great importance of these histopathological changes in disease diagnosis in Howler monkeys.

A pronounced negative correlation between apoptosis of hepatocytes and inflammatory mononuclear cell infiltration in the liver suggests significant YF histopathological differences between Howler monkeys, other primate species and humans. Indeed, in humans liver inflammation and hemorrhage are generally mild or absent in YF and considered important histopathological diagnostic criteria^{(5) (6) (7)}. Liver inflammation appears to be a significant change in YF-infected Howler monkeys and is not associated with hepatocyte apoptosis, which can mislead disease diagnosis. These findings suggest that different primates may exhibit a different response pattern to YFV infection and therefore requires a more careful diagnosis.

Hepatocyte apoptosis, previously described as Councilman body formation, is considered one of the most important and characteristic hallmarks of YFV infection^{(5) (6) (8) (10)}. These pathological changes are usually intense and occur in combination with midzonal necrosis. The inflammatory response is generally quite mild or absent and does not correspond to the extent and severity of hepatic injury and this may be in conjunction with over expression of tumor necrosis factor beta (TNF- β), even with the presence of hepatocyte necrosis^{(6) (8)}. However, a considerable number of YFV-infected Brazilian Howler monkeys presented hepatic inflammatory mononuclear cell infiltration and hemorrhage during epizootics in Brazil. YFV virulence is considered highly dependent on the virus strain and host species susceptibility⁽⁴⁾. Differences in liver histological patterns during naturally acquired YFV infection have been observed elsewhere^{(4) (8)}.

More recently, hypotheses have been proposed for the complex pathophysiology of YFV liver injury. In addition to the cytopathic effect of YFV, hypoxia due to vascular injury, the action of numerous cytokine and an intense cytotoxic response play an important role in the pathogenesis of YF. Together these insults are responsible for major liver injury^{(7) (8)}. Hemorrhagic diathesis in the midzonal region may reflect injury severity and vascular (endothelial cell) involvement in YF infection⁽¹⁷⁾.

Hepatic steatosis was frequently observed in YF-infected monkeys during the period evaluated. This is a common finding reported in both necrotic and preserved cells in YF infection^{(3) (5) (6)}. The presence of hepatocyte lipid vacuoles is almost constantly found in human YF cases, and this finding is also common in experimental models with other species infected with YFV^{(10) (16)}. Hepatic steatosis was observed in fewer than half of YF-infected Howler monkeys in contrast with human cases. This may indicate differences in the pathogenesis of YF infection in NHPs.

Renal acute tubular necrosis was observed in approximately 60% of infected animals in Brazilian YF epizootics, followed importantly by interstitial nephritis. These changes have previously been described in natural or experimental YF primate infection^{(2) (9)}. In the final stages of YFV infection, hypotension is considered responsible for oliguria and acute tubular necrosis development⁽¹⁾. In *Rhesus monkeys*, YFV exhibits strong viscerotropism causing severe kidney injuries, promoting hemodynamic changes, azotemia and organ failure⁽⁹⁾. In Howler monkeys, YFV seems to cause renal lesions similar to those observed in humans, and due to their high frequency they are almost certainly involved in the disease pathogenesis and mortality rates.

Major changes found in the spleen, heart, and lungs are not always directly associated with YFV infection, although hemorrhage in these organs is an expected change in YF-infected animals, which may result in bleeding diathesis⁽¹⁾. These changes

may reflect the splenic response to viral infection, however necrosis of the lymphoid follicles that usually occurs in YF was not observed⁽¹⁾⁽²⁾. Spleen, heart, and lung changes had a lower frequency than those registered in the liver and kidneys, and did not allow determination of the real importance of these organs in the diagnosis of YF in Brazilian Howler monkeys.

The determination of the frequency of histopathological changes in Howler monkeys naturally infected with YF in Brazil allowed demonstration of the similarities and differences of these alterations between NHPs and humans. This study has demonstrated that a significant proportion of Howler monkeys displayed hepatic inflammatory mononuclear cell infiltration and hemorrhage, which are usually criteria that may exclude YF diagnosis in human cases. Experimental studies with YF infection in *Rhesus monkeys* may present different morphological changes when compared with natural infection in Howler monkeys.

Howler monkeys play an important role in the YF epidemiological cycle in South America, and consequently more accurate morphological diagnostic criteria need to be established. This is particularly important considering the differences that may exist between the genera of Brazilian NHPs involved in YFV epizootics.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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