Original Article

Assessment of a diagnostic algorithm for yellow fever in non-human primate samples referred to the Pathology Center at the Adolfo Lutz Institute

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ABSTRACT

The Brazilian Health Department accredits the Pathology Center at the Adolfo Lutz Institute (CPA-IAL) as a macro-regional reference laboratory for yellow fever (YF) epidemiological surveillance in humans and non-human primates (NHP) in Brazil. CPA-IAL performs histopathological and immunohistochemical (IHC) analysis. Until 2018, all NHP samples for YF research were tested in both. In 2019, they implemented a screening-based diagnostic algorithm using histopathological features seen in liver tissue samples, thus carrying out more rationalized IHC tests. **Objective:** Assess the use of the diagnostic algorithm compared with the period before it was implemented. **Methods:** Retrospective study of NHP anatomopathology reports issued from 2018 to 2019 at CPA-IAL, in order to establish diagnosis performance indices of the histopathological test for YF epidemiological surveillance; to perform a sensitivity analysis of immunohistochemical test for samples with moderate to intense autolysis; and to compare the median time required for releasing reports for each period. **Results:** There was no statistically significant difference in histology and IHC performance for detecting YF between the pre and post-algorithm periods; there was an important reduction in the number of requested IHC tests, as well as in the time span for releasing reports ($p < 0.0001$). **Conclusions:** The algorithm showed similar performance, resulting in reduced due time for its releasing to epidemiologic surveillance, and in a reduced number of IHC reactions, therefore being proper for diagnosing yellow fever in NHP at CPA-IAL.

KEYWORDS: Algorithm, Surveillance, Yellow Fever, Primates, Zoonosis, Public Health.

RESUMO

O Centro de Patologia do Instituto Adolfo Lutz (CPA-IAL) é credenciado pelo Ministério da Saúde como laboratório de referência macrorregional para a vigilância epidemiológica de febre amarela (FA) em seres humanos e primatas não humanos (PNH) do Brasil, atuando por meio de análise histopatológica e imuno-histoquímica (IHQ). Até o ano de 2018, ambos os exames eram aplicados a todas as amostras de PNH recebidas para a pesquisa de FA. Em 2019, implantou-se um algoritmo diagnóstico baseado na triagem pelas características histopatológicas observadas no tecido hepático, possibilitando a racionalização do uso da IHQ. **Objetivo:** Avaliar a aplicação do algoritmo diagnóstico comparado ao período que antecedeu sua implantação. **Métodos:** Estudo retrospectivo de relatórios anatomopatológicos de PNH emitidos, entre 2018 e 2019, no CPA-IAL para determinação de índices de performance diagnóstica do exame histopatológico na vigilância epidemiológica de febre amarela,

avaliação da sensibilidade do exame imuno-histoquímico para amostras com autólise de moderada a avançada e comparação da mediana de tempo decorrido para emissão dos relatórios em cada período. **Resultados:** Não houve diferença estatisticamente significante na performance da detecção de FA por histologia e IHQ entre os períodos pré e pós algoritmo; houve importante redução na quantidade de exames IHQ solicitados e no tempo de liberação dos relatórios (p<0,0001). **Conclusões:** O algoritmo resultou em desempenho semelhante, redução do tempo de liberação oportuno para a vigilância epidemiológica do agravo e da quantidade de reações IHQ realizadas, portanto, apresentando-se adequado para o diagnóstico de febre amarela em PNH no CPA-IAL.

PALAVRAS-CHAVE: Algoritmo, Vigilância, Febre Amarela, Primatas, Zoonose, Saúde Pública.

INTRODUCTION

The yellow fever (YF) is a zoonotic, hemorrhagic, not contagious disease caused by an arbovirus of the *Flavivirus* genus, which is endemic in African, Central-American, and South-American tropical countries. It has already spread throughout countries like USA, Spain, France, England, and Italy.[1,2] In Brazil, it is a significant public health affliction.[3]

Two cycles of that arbovirus are known in the Brazilian territory: sylvatic and urban. In the urban cycle, which has been controlled since 1942, the virus transmission to humans happens through the *Aedes aegypti*[4] mosquito; in the current sylvatic, or jungle cycle, dissemination involves mainly mosquitoes of the *Haemagogus* and *Sabethes* genera. In Brazil, non-human primates (NHP) sustain the sylvatic cycle by amplifying the virus.[5,6]

Both humans and NHP are susceptible to the disease.[3] Therefore, non-human primates are a sentinel group for yellow fever epizootic surveillance, anticipating risk to human cases.[7] Sylvatic yellow fever outbreaks have been noticed to precede and follow human epizootics.[8,4] Neotropical primates comprise a broad group of 174 known species (216 species/subspecies), 42.1% of which are under threat.[9] In highly sensitive species to the virus, such as primates of the *Alouatta* genus–whose populations in different locations are severely affected by YF outbreaks–, monitoring the viral dissemination is vital, as well as drawing up strategies to conserve threatened species.[10]

Until 1999, Brazilian YF surveillance only studied human cases. Since 2000, the Brazilian Department of Health has incorporated the surveillance of the virus in NHP into the Yellow Fever Surveillance, Prevention and Control Program, by researching illness

and deaths in non-human primates throughout the country. Health professionals conduct such research using data from epizootic reports in the Information System for Aggravation Notification (Sinan), followed by material collecting for laboratorial diagnosis.[5,11]

The Adolfo Lutz Institute (ALI) – linked to the Disease Control Coordination, in the São Paulo State Health Department (DCC/SHD-SP)–works with laboratorial surveillance of YF in both humans and NHP, using serological tests; anatomopathological, immunohistochemical (IHC), and molecular (RT-qPCR and viral sequencing) analyses; and viral isolation of samples. Their Pathology Center (PC) performs histopathological tests on formalin-fixed samples of various biologic tissues–liver, spleen, kidney, heart, lung, and brain–, as well as IHC and RT-qPCR tests on paraffin-embedded liver samples. Histopathological analysis consists in identifying tissue changes at microscopic levels.[13,14] IHC is a complementary test to that analysis, whose objective is to microscopically locate specific antigens in the examined tissue samples through an antigen-antibody reaction.[15]

Until 2018, all formalin-preserved NHP samples sent to PC-ALI were submitted to IHC, no matter the histopathological findings. Despite its being an extremely important diagnosis tool, the IHC test incurs additional cost, hence the reason it is recommended as a confirmatory histopathology test.

The recent yellow fever epidemic in Brazil has broadened the knowledge of histological lesions, immunolabeling standardization, viral load, and susceptibility of Neotropical non-human primate groups. With the public contingency plan aiming for rapid completion of the studies and a quick response from the "Prof. Alexandre Vranjac" Epidemiological Surveillance Center (ESC/DCC/SHD-SP), PC-ALI have developed and implemented, in 2019, a diagnostic algorithm for systematizing immunohistochemical tests, promoting diagnosis efficiency in the context of epidemiological surveillance of YF in NHP.

This paper aims to evaluate the implementation of that algorithm by confirming accordance between histopathological results and immunohistochemical analysis; by ascertaining the performance of the tests; and by assessing the median time for releasing reports before and after the algorithm was implemented.

METHODOLOGY

Sampling

We have reviewed the non-human primate anatomopathology reports issued by the Laboratory Information Management System between June 2018–prior to the implementation of the algorithm–and April 2019–when the Pathology Center at the Adolfo Lutz Institute started using the algorithm in their NHP routine for YF research, in accordance with the National Program for Yellow Fever Epidemiological Surveillance.

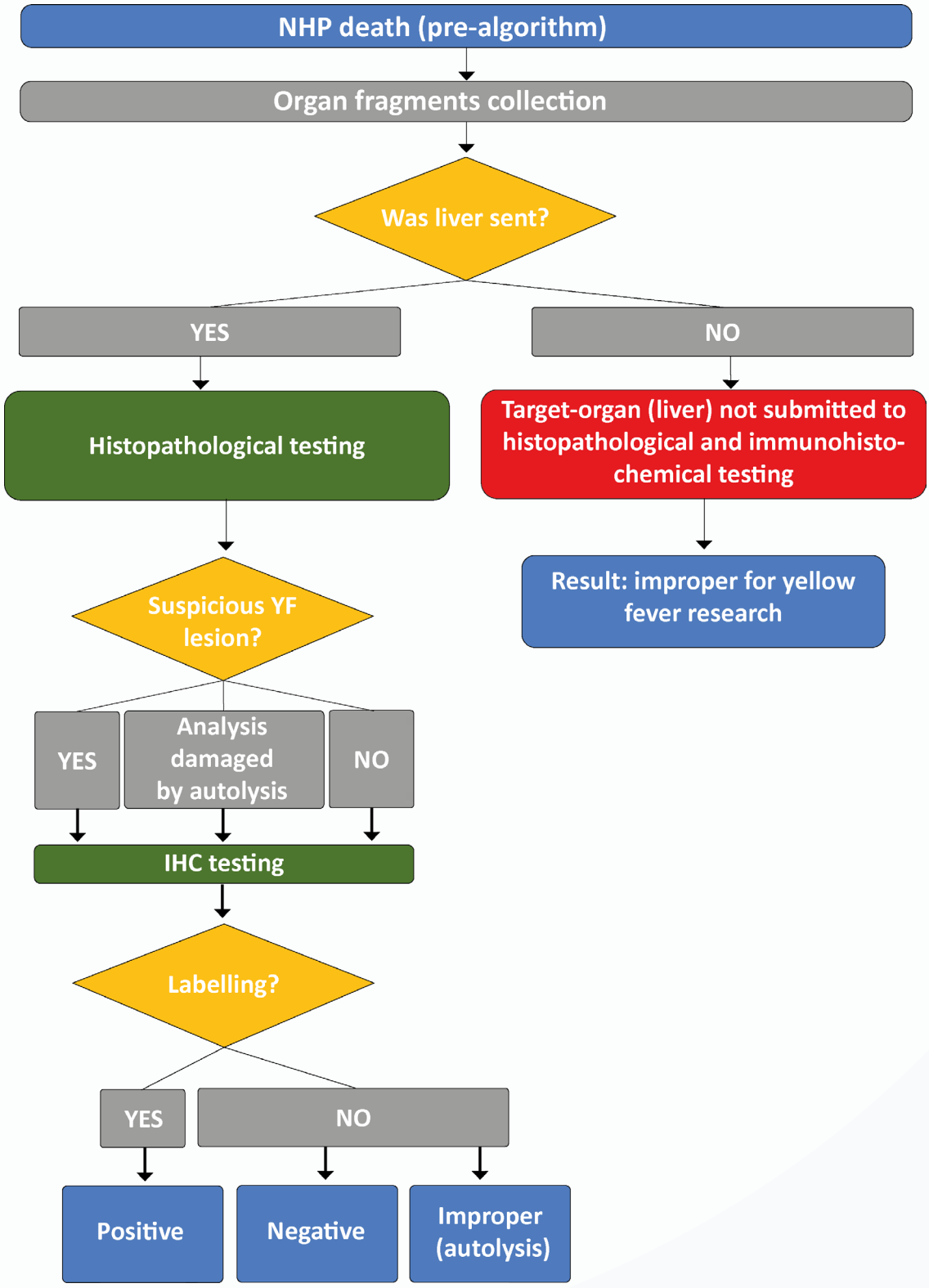
In order to evaluate the efficiency of the diagnostic algorithm, we have selected 400 cases: 200 reports prior to its implementation, and 200 reports from the subsequent period. To achieve the proposed goal, we have screened 546 NHP anatomopathology reports, excluding 146 cases. The chosen criteria for selecting cases for this study were the mandatory submission of 10% formalin-preserved liver for histopathological analysis, with mild to absent autolysis, allowing proper morphological assessment of microscopic changes; and RT-qPCR testing on fresh frozen liver samples.

We have verified and analyzed the information regarding the non-human primate genera, the histopathological and immunohistochemical descriptions of liver fragments provided by the Pathology Center at ALI, and the results of RT-qPCR molecular testing provided by the Virology Center at ALI.

Algorithm

Prior to the implementation of the diagnostic algorithm for YF, all PNH liver samples referred to the Pathology Center were necessarily submitted to histopathological analysis, to morphologic description of tissue changes at both microscopic and immunohistochemical levels, and to research into yellow fever virus antigens in tissues, regardless of what kind of changes were found in the histopathological test. Only when liver fragments were not referred, the tissue samples were histopathologically tested; but such cases were considered improper for virus research, and not submitted to immunohistochemical studies. Figure 1 illustrates the diagnosis flow we followed in that period.

Figure 1. Diagnosis flow of NHP samples for YF surveillance in the Pathology Center at Adolfo Lutz Institute, in the period prior to the implementation of the diagnostic algorithm, in 2019.



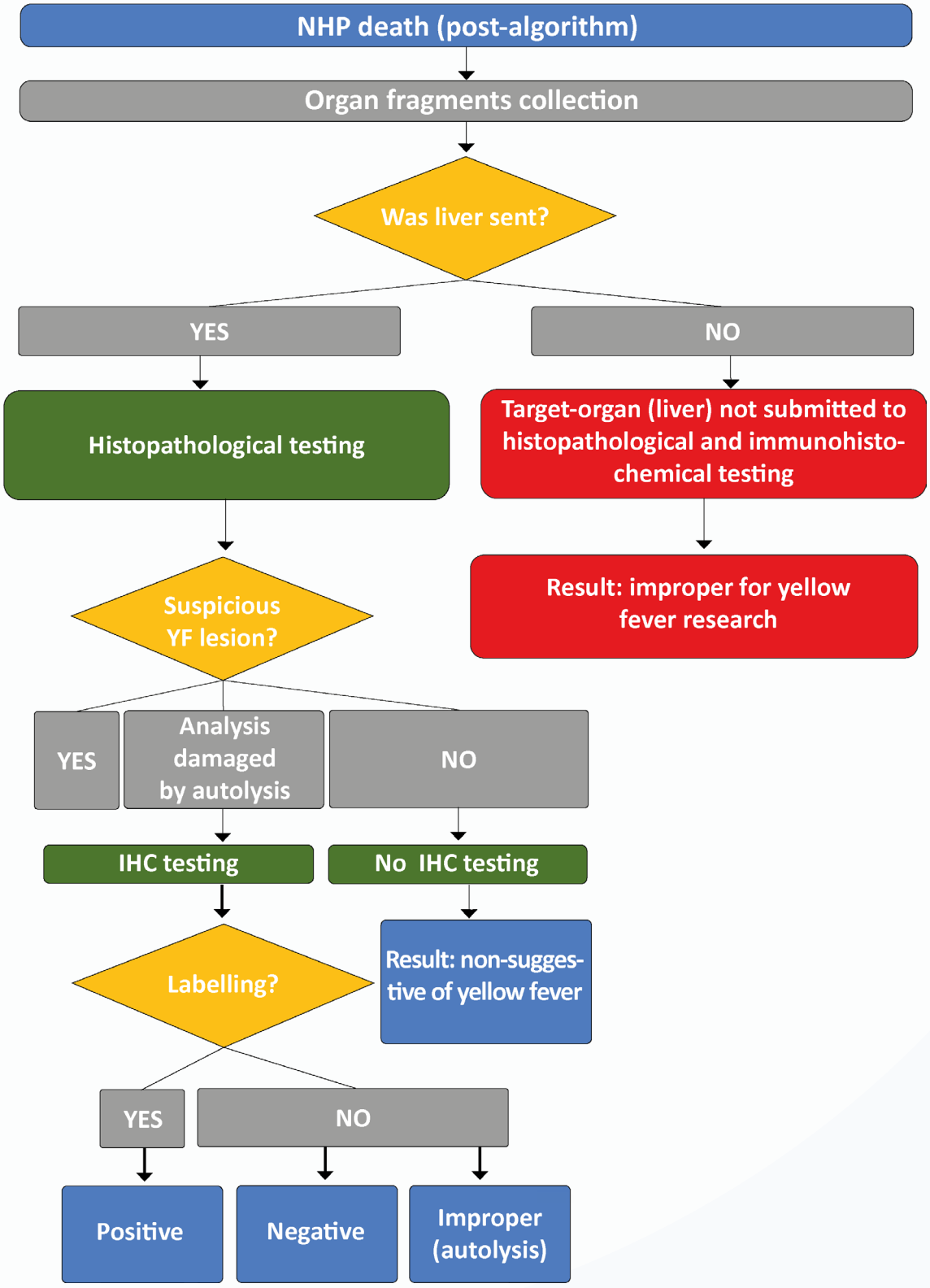
With the algorithm, the liver samples started to be screened using the pattern of microscopic tissue changes. The ones included in the scope of lesions caused by the yellow fever virus were referred to virus antigen research by ancillary immunohistochemical test. Table 1 shows the suspicious histopathological findings.

With the implementation of the algorithm, four categories were established for the non-human primate samples. Figure 2 illustrates them.

Table 1. Histological lesions seen in YF virus infection in NHP.

	Suspicious histological findings
Classic findings	Hepatic: massive midzonal to panlobular hepatocyte necrosis/apoptosis, with presence of hyper-eosinophilic apoptotic corpuscles (Councilman-Rocha Lima).
Frequent findings	Hepatic: scarce, predominantly mononuclear inflammatory infiltrate; macro and microcytic steatosis, especially the morula-form pattern one. Spleen: lymphoid depletion.
Occasional findings	Hepatic: hemorrhage and hemosiderosis. Kidney: acute tubular necrosis, intratubular proteinaceous material. Lung: intra-alveolar hemorrhage

Figure 2. YF diagnosis flow in NHP after the implementation of the algorithm, in 2019, in the Pathology Center at Adolfo Lutz Institute.



Statistical analysis

We have tabulated and analyzed the results from the histopathological, immunohistochemical, and RT-qPCR tests on fresh samples. We have calculated the following performance indices: sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the laboratory tests used to research yellow fever in the pre and post-algorithm periods, using the MedCalc Software. We have calculated the Kappa value of accordance with the RT-qPCR molecular result using the software GraphPad, San Diego, California (USA), whose result was considered as gold standard. To calculate performance and accordance, we have considered, in the pre-algorithm period: as positive, the cases with positive IHC; and negative, the cases with negative IHC. In the post-algorithm period: positive, the cases with positive IHC; and negative, the cases with non-suggestive histology of yellow fever, and the cases with negative IHC.

To measure the due time for releasing the results, we have calculated the statistical significance based on the median time of each period, using the Wilcoxon statistical test, with the MedCalc software.

RESULTS

In the period prior to the implementation of the diagnostic algorithm for YF in NHP, the analyzed samples represented the following genera: 77% (154/200) *Callithrix*; 9.5% (19/200) *Alouatta*; 5% (10/200) *Sapajus*; 0.5% (1/200) *Callicebus*; and 8% (16/200) non-classified genus.

After histopathological analysis, immunohistochemical testing complemented the laboratorial studies of all cases, with 4% (8/200) positive results for virus antigens. RT-qPCR molecular testing was positive in 5% (10/200) cases. There was excellent accordance between the results from histopathological and immunohistochemical tests, and the results from RT-qPCR (κ = 0.884, CI 95%: 0.724 to 1,000). Such a study flow showed the following performance: 80% sensitivity (CI 95%: 44.39% to 97.48%); 100% specificity (CI 95%: 98.08% to 100.00%); 100% positive predictive value (PPV); 98.96% negative predictive value (NPV) (CI 95%: 96.49% to 99.70%); and 99% accuracy (CI 95%: 96.43% to 99.88%).

In the period subsequent to the algorithm, in 2019, the analyzed samples represented the following genera: 76% (152/200) *Callithrix*; 12.5% (25/200) *Alouatta*; 5% (10/200) *Sapajus*; 0.5% (1/200) *Callicebus*; 0.5% (1/200) *Ateles*; and 5.5% (11/200) non-classified genus. At that time, 17% (34/200) cases showed suspicious yellow fever lesions, and were

referred to immunohistochemical testing. The remaining cases (83%; 166/200) had no suspicious lesions, and were concluded.

Out of the cases using immunohistochemistry, 8.8% (3/34) tested positive for YF, while 2% (4/200) were positive in the RT-qPCR molecular test, which was applied in all samples. There was excellent accordance (κ = 0.855, CI 95%: 0.573 to 1.000) between the results from all tests and the results from the molecular test. After the algorithm, there was the following performance: 75% sensitivity (CI 95%: 19.41% to 99.37%); 100% specificity (CI 95%: 98.14% to 100.00%), 100% PPV; 99.49% NPV (CI 95%: 97.29% to 99.91); and 99.5% accuracy (IC 95%: 97.25% to 99.99). Table 2 compiles the performance indices of the set of laboratorial analysis used before and after the implementation of the algorithm. The estimated prevalence of the disease in the studied period remained constant.

The time of laboratorial research in the periods prior and subsequent to the implementation of the algorithm–from the sample arriving at ALI to the releasing of its result in the Internal Hospital Management System (SIGH)–was represented by a median of 17 (from 5 to 52) and 13 (from 2 to 34), respectively (p <0.0001).

Samples excluded from the algorithm analysis represented 10.4% (57/546) cases with moderate to intense hepatic autolysis degree, out of which 3.5% (2/57) tested positive in immunohistochemical analysis, confirmed by molecular testing. The other tests were considered improper, and the molecular analysis was negative.

Tabela 2. Performance do conjunto de análises laboratoriais utilizados antes e após a implantação do algoritmo.

	Pre-algorithm	Post- algorithm
Kappa accordance coefficient of laboratorial analysis with RT-qPCR result	κ = 0.884 (CI 95%: de 0.724 to 1.000)	κ = 0.855 (IC 95%: 0.573 a 1,000)
Sensitivity	80% (IC 95%: 44.39% a 97.48%)	75% (IC 95%: 19.41% a 99.37%)
Specificity	100% (IC 95%: 98.08% a 100.00%)	100% (IC 95%: 98.14% a 100.00%)
Positive predictive value	100%	100%
Negative predictive value	98.96% (IC 95%: 96.49% a 99.70%)	99.49% (IC 95%: 97.29% a 99.91%)
Accuracy	99% (IC 95%: 96.43% a 99.88%)	99,5% (IC 95%: 97.25% a 99.99%)
Median time for releasing reports, in days *	17	13

* p< 0.0001.

DISCUSSION

Epizootic surveillance is a significant factor in prevention and control plans for diseases of public health relevance. It is useful to early detection and timely action, as well as in monitoring and evaluating interventions.[16] Carrying out a proper surveillance demands constant and systematic collection, assessment, data interpretation, and integrated dissemination of the achieved results to officers responsible for disease prevention and control measures.[17,18]

The histopathological test is a valuable tool for infectious disease surveillance programs. It consists in the microscopic analysis of tissue morphology, allowing the identification of abnormalities. Therefore, it is considered a screening test; the identification of injury patterns leads to the diagnosis, or to the ascertainment of etiological suspicions.[14] For certain infectious agents, suspicions confirmed by histopathological test need to be complemented by other analysis; immunohistochemical and PCR molecular tests are mostly frequent in diagnostic routines.[13,14]

Brazil has a great diversity of non-human primate genera, amongst which there are different susceptibilities to YF virus infection, and to death from acute liver failure. The *Alouatta* genus is the most susceptible to death by such a viral infection; histopathological testing on livers has shown a wide range of lesions, such as hepatocyte necrosis and apoptosis with presence of Councilman-Rocha Lima corpuscles, macro and microcytic steatosis, and hemorrhage. The *Callicebus* and *Sapajus* genera are also susceptible, having developed similar hepatic lesions. The *Callithrix* genus was the largest sampling group at the Adolfo Lutz Institute during the 2017 yellow fever outbreak. However, that group presented the lowest proportion of positive cases and the lowest viral load when compared with the *Alouatta* genus. It was also the main group with positive discordant animals – they were defined as positive by PCR virus detection, but had no histological lesions and no detected viral antigen by immunohistochemistry in liver tissues.[12]

The sensitivity, both pre and post-algorithm, may have been influenced by the occurrence of positive discordant cases. Finally, in the group excluded due to autolysis, there were cases with detectable antigen, demonstrating the importance of IHC testing on tissues under those conditions.

With the achieved results, therefore, the diagnostic algorithm for yellow fever in NHP in the Pathology Center at IAL has revealed itself as an interesting diagnosis alternative, as it keeps equivalent diagnosis ability to the period prior to its implementation, while reducing significantly the number of required immunohistochemical tests, the costs and the due time for laboratorial research completion. That fact has allowed time and resources to be

relocated for studies on other diseases of public health interest, and for conserving wildlife, which is a significant aspect in the context of health work. For a better quality in using the algorithm, we reinforce the importance of a multidisciplinary team including veterinary pathologists with know-how in wildlife pathology, especially non-human primates.[14]

CONCLUSION

The diagnostic algorithm has shown similar performance to the previously used model, being suitable to the diagnosis routine of yellow fever in NHP. Additional benefits were a reduced number of required immunohistochemical tests, and a shorter time span for releasing research reports so that the ECP/DCC-SP's epidemiological surveillance can perform prevention and control measures in a due time.

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Historic

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