Pulmonary granuloma is not always the tuberculosis hallmark: pathological findings of different tuberculosis stages in New and Old World Nonhuman Primates naturally infected with the *Mycobacterium tuberculosis* Complex

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Abstract

Herein we present the pathological findings of different tuberculosis stages in Old and New World monkeys kept under human care in Rio de Janeiro, Brazil and naturally infected with *Mycobacterium tuberculosis* Complex. Fifteen nonhuman primates from five different colonies were incorporated into the study. There are 60% (9/15) Old World Monkeys and 40% (6/15) New World Monkeys. According to the gross and histopathologic findings, the lesions in nonhuman primates of this study are classified into the chronic-active, extrapulmonary, early-activation or latent-reactivation tuberculosis stage. Among the Old World Monkey, 66.7% (6/9) of nonhuman primates, all rhesus monkeys (*Macaca mulatta*), showed severe granulomatous pneumonia. In all Old World Monkeys cases, typical granulomas were seen in at least one organ regardless of the stage of the disease. In the New World Monkeys, the typical pulmonary granulomas were seen in 16.7% (1/6) of the cases, just in the latent-reactivation stage in Uta Hick's Bearded Saki (*Chiropotes utahickae*). In this study, 66.7% (6/9) of Old World Monkeys (OWM) and 83.3% (5/6) of New World Monkeys (NWM) showed pulmonary changes at the histological evaluation. The tuberculosis diagnosis in the nonhuman primates in this study was based on pathological, immunohistochemical, molecular, and bacteriological culture. Although the typical presentation was observed in some cases, the absence of pulmonary granuloma did not exclude the tuberculosis occurrence in nonhuman primates of the Old and New World. Tuberculosis should be included as a cause of interstitial pneumonia with foamy macrophages infiltration in the New World nonhuman primates. Due to the high sensitivity of immunohistochemistry with Anti-*Mycobacterium tuberculosis*, we suggest the addition of this technique as a diagnostic tool of tuberculosis in the nonhuman primates even when the typical changes are not seen.

1. Introduction

Mycobacterium tuberculosis complex (MTBC) refers to a group of mycobacteria encompassing nine members of closely related species that cause tuberculosis (TB) in animals, including humans (Kanabalan et al., 2021). Tuberculosis is consistently the most impactful bacterial disease to affect humanity, with a quarter of all humans infected and responsible for the most significant number of infection-related deaths and long-term disability (WHO, 2020a). In 2019, an estimated 10 million people were having health issues with TB worldwide, and it was considered one of the top 10 causes of human death and the leading cause of a single infectious agent (WHO, 2020b). Tuberculosis is a chronic airborne disease that causes high morbidity and mortality in humans and nonhuman primates (NHPs), especially captive macaque monkeys (Warit et al., 2020).

Although particular species may be more or less susceptible to disease, all nonhuman primates can develop tuberculosis (Mätz-Rensing and Lowenstine, 2018). Due to the high genetic similarity with humans, NHPs accurately model all TB aspects and are considered the gold standard biomodel in experimental *M. tuberculosis* infection (Foreman et al, 2017). Infections in captive nonhuman primates by MTBC mainly occur due to direct contact with TB-infected humans through the inhalation of aerosolized bacteria (Ghodbane and Drancourt, 2013; Mätz-Rensing et al, 2015; Warit et al., 2020). Captive nonhuman

primates develop an infection clinically indistinguishable from human tuberculosis (Kaushal et al., 2012; Kanabalan et al., 2021), and they recapitulate the full spectrum of infection outcome and pathology seen in humans (Scanga and Flynn, 2014).

The hallmark of tuberculosis is a typical granuloma presentation in any organ (Mätz-Rensing and Lowenstine 2018). According to Lin et al. (2009), in macaques with active-chronic TB, classical caseous granulomas, suppurative granulomas, nonnecrotizing granulomas, tuberculous pneumonia, and cavitary lesions were observed. The authors report latently infected monkeys present classic caseous lesions with mineralized granulomas and completely fibrotic pulmonary areas. In cases of rapidly progressive TB, the authors report a pattern of lesions consistent with a primary pulmonary disease with disseminated miliary, sometimes confluent small pulmonary granulomas, and extrapulmonary spread into the hepatic, splenic, and mesenteric tissue.

TB is well known and widely reported disease in laboratory Old World Monkeys (OWM), and most experimental studies have been performed in this nonhuman primate group (Schmidt 1956; Clarke, 1968; Walsh et al. 1996; Okada et al. 2007; Reed et al. 2009; Mehra et al. 2013). The natural occurrence in New World Monkeys (NWM) species is considered uncommon (Mätz-Rensing and Lowenstine 2018). Reports of Brazilian TB occurrence in nonhuman primates are scarce. Brazil is among the 30 countries with the highest incidence of human tuberculosis worldwide (WHO, 2019) and in 2020, Rio de Janeiro state was considered the second state with the most cases of human tuberculosis (BRASIL 2020). We describe the pathological findings of different stages of *Mycobacterium tuberculosis* Complex infection in New and Old World Nonhuman Primates kept under human care in Rio de Janeiro, Brazil.

2. Materials And Methods

2.1 Animals and study area

This study was conducted in nonhuman primates kept under human care in Rio de Janeiro, Brazil. Fifteen nonhuman primates with a suspected or prior diagnosis of tuberculosis, regardless of species, sex or age, from five captive colonies were included in the study. The epidemiological data about sex and age in each case were acquired with the colonies' technical veterinary.

The colony is the Nonhuman Primate Breeding Service (SCPrim) of the Biomodel Science and Technology Institute (ICTB) of the Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, Brazil (2°52'39.06" S, 43°14'46.20" W). Currently, SCPrim has 510 rhesus monkeys (*Macaca mulatta*), 56 cynomolgus monkeys (*Macaca fascicularis*) and 123 squirrel monkeys (*Saimiri* sp.), of which 118 are *S. sciureus* and five *S. ustus*. The nonhuman primates are bred and maintained according to the guidelines of the National Council for the Control of Animal Experimentation, CONCEA (BRASIL, 2015), the Guide for the Care and Use of Laboratory Animals (National Research Council, NRC, 2003; 2011) and the Federation of European Laboratory Animal Science Associations (FELASA) (BALANSARD et al., 2019). Animal handling procedures were approved by the Ethics Committee on the Use of Animals (CEUA, Fiocruz) under license

number LW-5/16 and additive terms. The management of wild fauna in the farm is also authorized by the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA) under number 556664, INEA-RJ.

All colonies receive filtered and treated water *ad libitum* through automatic stainless steel drinkers. The diet consists of extruded commercial feed (Nuvilab® Old World Monkeys and Nuvilab® Neotropical Primates, both from Quimtia, Colombo, Paraná, Brazil) in the morning, supplemented with horticultural items in the afternoon. The vegetables are routinely sanitized by submersion in a chlorinated solution at 0.02% for 20 minutes for parasitological and bacteriological control. The enclosures, built-in grid and masonry, are furnished with perches, drums and swings. Other food, physical or sensory environmental enrichment items are offered alternately (grains, herbs, fruit ice cream, popcorn, tire swing, swimming pool on hot days, among others). All enclosures have a covered refuge area for animals to protect themselves from weather and other animals during a fight. The enclosures included an open environment with natural light and trees.

The rhesus colony has nine modules divided into two enclosures (A and B) with 64m² each and two modules divided into four enclosures (A, B, C and D) with 34m² each totaling 26 enclosures. In these enclosures, groups of five NHP on average are housed. Some enclosures house all-male groups, and there is an enclosure for older NHP. The monkeys are divided into family groups consisting of male and female alphas and other animals of different ages, with an average of 20 individuals per enclosure. The smaller cages house the primates or groups of all males separated to carry out population control of the colony or reserve breeding stock.

The cynomolgus colony is bred in a building consisting of five in-line enclosures measuring 7.35 m² each, containing part of the covered area and part of the uncovered area. The social groups are formed by males, females of different ages and offspring, with an average of 11 individuals per enclosure. There is also a group composed only of males, with 15 NHP. The Saimiri colony forms a complex of six modules divided into two enclosures (A and B) with an external area of 17.10 m² and an internal area (refuge) with 2.85 m², a total of 19.95 m² each. Social groups are distributed with an average of 11 animals per enclosure.

The colony of the Triage Center of Wild Animals (CETAS), in Seropédica, Rio de Janeiro state, Brazil (22°43'34.1"S 43°42'22.4"W). CETAS is a federal environmental organization that works with wildlife animal rescue, especially animals from smuggling. The goal is to rehabilitate and reintroduce many species in their natural habitat or redirect them to a definitive place, such as a zoo or a legal breeder. The center works approximately with 6,000 animals a year and is under the jurisdiction of the IBAMA. Installation of CETAS at approach time housed about 10 *Sapajus* sp. and 14 *Callithrix* sp., but that number changes drastically once the center receives and destines animals every once in a while, as well as there could be deaths for many different reasons. The primates in the center mainly eat fruits, vegetables, boiled eggs and raw cattle hearts previously frozen. These items are washed with common

sink water, and there is no control of the quality of this water. Because the center has limited money resources, it is not uncommon for the primates to be fed a limited variety of items for long periods.

The *Sapajus* spp. are kept in an exclusive block for larger animals, a medium-size enclosure 3 x 3,44 x 4 m has four in-line smaller enclosures inside, five on each side. Inside each enclosure, the monkeys are kept according to affinity and proportion of males to females (approximately 1:2). In this study, there was no breeding control program, and all animals were intact. Inside their enclosure, there are perches (varying in size), as well as strings and hanging tires for their entertainment. There are no trees and/or plants.

The *Callithrix* sp. species are kept in individual bird cages or into families in a cement enclosure in a block composed of a line of eight enclosures. They are surrounded by psittacines and passerines' enclosures (on the right and the left, respectively). Each enclosure has about 17 m². These NHP have no tagging or any other type of marking, so it is almost impossible to track their origin once they arrive in the center after a while. In cases in which there are males and females together, the males are castrated. CETAS is short in staff, and the same keeper that handles the other animals (birds, reptiles and mammals) also handles the *Callithrix* and *Sapajus* species.

The colony of the Rio de Janeiro Primatological Center (CPRJ) of the Environmental State Institute (INEA) of Rio de Janeiro, Brazil (22°29'14.51" S, 42° 48'78" W), an institution devoted to the Conservation of endangered neotropical primates. The nonhuman primates are bred and maintained according to the CONCEA (BRASIL, 2015) and the Best Practice Guidelines for Callithrichidae, EAZA (AMSTERDAM, 2017). The breeding colony is authorized by the IBAMA, under number 4989806, INEA/RJ.

Currently, CPRJ has 350 neotropical primates, 276 of which are from the Callithrichidae family (genus *Callithrix*, *Cebuella*, *Mico*, *Saguinus* and *Leontopithecus*), 41 from Cebidae (genus *Sapajus*, *Saimiri* and *Cebus*), 3 Aotidae (genus *Aotus*), 15 Pitheciidae (*Callicebus*, *Plecturocebus*, *Pithecia*, *Cacajao* and *Chiropotes*) and 12 from Atelidae (genus *Alouatta* and *Ateles*). These primates are housed in family groups or pairs, with no public access, in 140 outdoor enclosures built in masonry and iron screen and furnished with perches and nest boxes or shelters. The enclosures are located in an open environment with access to natural sunlight and are protected from rain and cold wind. The CPRJ possesses an area of 270 hectares in the Parque Estadual dos Três Picos (PETP) in the Bioma Atlantic Forest.

The primates receive treated water *ad libitum* in stainless steel drinkers. The diet consists of extruded commercial feed (Nuvilab® Old World Monkeys and Nuvilab® Neotropical Primates, both from Quimtia, Colombo, Paraná, Brazil) in the morning, supplemented with horticultural items in the afternoon. Folivorous species also receive fresh leaves every day, and the insectivorous receive bugs and worms regularly. The vegetables are routinely sanitized by submersion in a chlorinated solution at 0.02% for 20 minutes for parasitological and bacteriological control.

2.2 Clinical Assessment

At Nonhuman Primate Breeding Service (SCPrim/ICTB), the NHPs are physically restrained and sedated with an association of ketamine hydrochloride (Cetamin, Syntec®, Brazil) 10 mg/kg + midazolam hydrochloride (Teuto®, Brazil) 1mg/kg intramuscularly. After restraint, the primates are weighed, and clinical examination is performed, such as palpation of the abdomen and lymph nodes, examination of the oral cavity, skin, mucous membranes, pulmonary and cardiac auscultation, and temperature verification. Depending on the case, blood is collected for hemogram and biochemistry, sent to the ICTB laboratory.

In the Triage Center of Wild Animals (CETAS), in order to perform clinical and routine examinations, the *Callithrix* species are manually restrained (usually by towels or leather gloves), and *Sapajus* species are examined under sedation with ketamine + midazolam intramuscularly (the dose varying from 10mg/kg to 0,5mg/kg to 1mg/kg, respectively). These animals are also weighed, palpated and checked for abnormalities in heart/lungs auscultation, oral cavity, lymph nodes, skin, mucous membranes and temperature. Clinically ill patients can have blood withdrawn, feces collected, and x rays/ultrasound performed at the Veterinary Hospital at the UFRuralRJ.

At CPRJ, the NHP are captured with nets and physically restrained and sedated with ketamine hydrochloride (Cetamin, Syntec®, Brazil) 10 mg/kg + midazolam hydrochloride (Teuto®, Brazil) 1mg/kg intramuscularly. After restraint, the primates are weighed, and clinical examination is performed, such as palpation of the abdomen and lymph nodes, examination of the oral cavity and teeth, nostrils, ears, eyes, skin and fur, mucous membranes, genital, urinary and rectal openings, pulmonary and cardiac auscultation and temperature verification. Clinically ill animals can have biological samples analyzed or x-rays/ultrasounds performed.

2.3 Necropsy and sample collection

In the cases of death or euthanasia in nonhuman primates, the necropsy was realized by the technical responsible of the colony. The euthanasia of nonhuman primates from SCprim colonies was performed after sedation with ketamine hydrochloride 10mg/kg + midazolam hydrochloride 1m/kg and consist of intravenous injection of thiopental sodium 30–60 mg/kg with continuous infusion until the deep plane of anesthesia is reached, then progressing to coma and death. In the case of nonhuman primate euthanasia from CETAS colony, the sedation was performed with ketamine 40mg/kg + xylazine 2mg/kg intramuscularly and, after the loss of consciousness and protective reflexes, subsequent intracardiac administration of potassium chloride 19,1%. These methods follow the CONCEA Normative Resolution 28/2015 and Brazilian Guide of Good Practices for Animal Euthanasia (CFMV 2013; BRASIL, 2015).

The necropsy of all nonhuman primates was performed by at least two veterinary pathologists of the Anatomy Pathology Sector of Federal University Rural of Rio de Janeiro (SAP/UFRuralRJ), Nonhuman Primate Breeding Service (SCPrim) or Primatology Center of Rio de Janeiro (CPRJ). During the procedure, personal protective equipment was used. A complete set of tissue samples as skin, skeletal muscle, tongue, thyroid, lymph nodes, trachea, lungs, heart, liver, spleen, kidneys, adrenals, stomach, intestines, pancreas, brain, and nerves were collected in each case and fixed in 10% buffered formalin solution.

Fragments from multiple organs were collected in microtubes for referral to bacterial culture (N = 6) and molecular biology (N = 14).

2.4 Histopathology and immunohistochemistry

The fragments stored in formalin will be fixed for 24–48 hours and subsequently submitted to routine histological processing. Histological slides were stained with Hematoxylin and Eosin and then observed under an optical microscope. Histological sections with lesions morphologically compatible with tuberculosis were submitted to the Ziehl-Neelsen histochemical technique for acid-alcohol-resistant bacilli evidence. To determine the fibrous capsule of granuloma, sections of the lung were submitted to Trichrome Masson's histochemical technique.

At the immunohistochemistry technique, blocks were cut into 3 µm slices and mounted onto silane-coated microscope slides. Antigen retrieval was performed with 0.1% trypsin for 30 minutes in an oven at 37°C. The sections were then incubated in methanol: H₂O₂ solution (97%:3%) for 30 min to block nonspecific binding. The sections were set at 37°C for 2 hours with primary anti-*Mycobacterium tuberculosis* polyclonal antibodies (Bioscience Inc®, San Diego, CA, USA) at 1 in 300 dilutions or phosphate buffer saline (PBS) as the negative control. As a positive control, a case previously confirmed by PCR will be used, and as a negative control of the reaction, the primary antibody will be replaced by Buffered Saline Solution (PBS). Then, the sections will be submitted to EnVision™ secondary polymer (Dako®, Carpinteria, CA, USA) in an oven at 37°C for 30 minutes. The development was done with 3,3'-diaminobenzidine chromogen (DAB + Substrate Chromogen System, DakoCytomation®, Carpinteria, California) for 2 minutes. Finally, the histological sections were counterstained with Harris hematoxylin and coverslipped. Were considered positive the cases in which structures morphologically compatible with *Mycobacterium* spp. bacilli were evidenced extracellularly or inside the cytoplasm of macrophages and multinucleated giant cells.

2.5 Molecular Analysis

The samples in RNA later were submitted to DNA extraction with glass beads, followed by phenol-chloroform, according to Sambrook and Russel (2001). In turn, the paraffin samples were extracted according to Shi et al (2004), adding glass beads. For the identification of CMTB, oligonucleotides INS1(5'-CGTGAGGGCATCGAGGTGGC-3') and INS2(5'-GCGTAGGCGTCGGTGACAAA-3') were used, which detect the genomic region IS6110 (Kolk et al., 1992). The amplified products were separated by electrophoresis in 1.5% agarose gel for 1 hour at 60 V, stained with Gel Red (Biotium) and visualized in ChemiDoc™ XRS using ImageLab™ software. For the identification of mycobacteria at the species level, oligonucleotides TB11 (5'-ACCAACGATGGTGTGTCCAT-3') and TB12 (5'-CTTGTCGAACCGCATACCCT-3') were used, which detect the one segment of the heat shock protein gene of 65 kDa (hsp65), according to Telenti et al. (1993). The amplified products were separated by electrophoresis in 1.5% agarose gel for 1 hour at 60 V, stained with Gel Red (Biotium) and visualized in ChemiDoc™ XRS using ImageLab™ software. The PCR product was purified in the GFX™ PCR DNA

and Gel Band Purification kit (GE Healthcare) and submitted to sequencing in the automatic ABI-PRISM 3500 Genetic Analyzer (Applied Biosystems).

For molecular diagnosis in live animals, oral swab, nasal swab, rectal and gastric lavage samples are collected. Swabs are collected without medium, and gastric and rectal lavage are performed with the aid of a probe and the use of 0.9% saline solution. Samples are kept refrigerated for up to two hours after collection and sent for laboratory analysis. After treatment and purification with NALC-NaOH (N-acetyl-L-cysteine-sodium hydroxide), the samples are analyzed using GeneXpert® (Cepheid, USA) commercial kits Xpert® MTB/RIF or Xpert® MTB/RIF Ultra, for MTBC complex detection and rifampicin resistance test.

2.6 Bacterial culture

Previously macerated tissues were cultured using the Ogawa-Kudoh method (Kudoh, Kudoh, 1974). All samples are also sent for direct smear microscopy with Ziehl-Nielsen staining to identify acid-resistant bacilli and bacterial culture in Löwenstein-Jensen medium and incubated for up to 72 days at 37°C in a biological safety cabinet class 3. The *Mycobacterium tuberculosis* complex species in positive cultures were identified using an immunochromatographic assay with the MPT64 marker (Silva et al., 2017).

2.7 Tuberculosis stage classification

To determine the tuberculosis stages as active-chronic and latent-reactivation, the criteria established were followed by Capuano et al. (2003) and Lin et al. (2009). We suggest classifying the early-activation stage of tuberculosis according to clinical and morphological findings. There was no evidence of necrosis or granuloma formation in the lung parenchyma or any other organ analyzed to fulfill the criteria for the early-activation stage. Cases in which typical tuberculosis granulomas, macro or microscopic, are seen in any organ, provided no pulmonary involvement, were considered extrapulmonary.

3. Results

2.1 Animals

Fifteen nonhuman primates from five different colonies in Rio de Janeiro, Brazil, were incorporated into the study. There are 60% (9/15) Old World Monkeys and 40% (6/15) New World Monkeys. The Old World primates belonged to the SCPrim rhesus and cynomolgus colony, seven rhesus monkeys (*Macaca mulatta*) and two cynomolgus monkeys (*Macaca fascicularis*). Among the rhesus monkeys, 71.4% (5/7) were male and 28.5% (2/7) female. The average age of rhesus monkeys was 5 years old. The two cynomolgus monkeys were female, with a mean age of 17 years old. Of the six New World primates, 50% (3/6) were marmosets (*Callithrix* sp.) from the CETAS colony, 33.3% (2/6) were squirrel monkeys (*Saimiri ustus*) from the SCPrim colony, and 16.7% (1/6) was Uta Hick's Bearded Saki (*Chiropotes utahickae*) from the CPRJ colony. Among the marmosets, 66.6% (2/3) were male, and 33.3% (1/3) were female. The marmosets were captured from free-ranging with no previous history. Although it was not possible to determine their exact age, the individuals exhibited sexual maturity. Fifty percent of the squirrel monkeys

were male and 50% female (1/2), with an average age of 6.5 year-old. The Uta Hick's Bearded Saki was male and aged 10 years. The sex and age of each nonhuman primate in this study are shown in Table 1.

Placeholder for Table 1; see Supplementary Files section.

2.2 Clinical Assessment

Regardless of disease stage, weight loss, anorexia and lethargy were the main clinical signs observed in the nonhuman primates with tuberculosis in this study. Clinical respiratory signs were observed in 55.5% (5/9) of cases in OWM and 16.7% (1/6) of cases in NWM. The summary of clinical findings in each case and other relevant clinical considerations are available in Table 1.

2.3 Gross findings

The changes and severity of macroscopic alterations of all nonhuman primates (NHP) with tuberculosis are available in Table 2. Among the Old World Monkey (OWM) group, 66.7% (6/9) of NHP, all rhesus monkeys, showed severe granulomatous pneumonia with well-demarcated structures, sometimes elevated, firm yellow-white or grayish nodules of different sizes, the typical gross pulmonary granulomas (Fig. 1). In all cases, single or coalescing nodules are seen in all pulmonary lobes, but the cranial lobes are most severely affected. The lungs are diffusely firm, enlarged and reddish with multiple consolidations areas. Sometimes, multiple cavitation areas of different sizes are seen on the airways (Fig. 1, inset). Ghon's complex presents all six cases with granulomatous pneumonia, with the mediastinal lymph node enlarged in different degrees.

Placeholder for Table 2; see Supplementary Files section.

In all cases of tuberculosis in OWM, typical granulomas were seen in at least one organ. The gastrointestinal, lymphatic, musculoskeletal, urinary and nervous systems showed multiple granulomas of variable size and severity and are the most affected systems of multisystemic tuberculosis presentation. In one case (NHP 6), a rhesus monkey presents multiple granulomas of the variable size seen in the brachial plexus nerves (Fig. 4). In this group, granulomatous lymphadenitis and granulomatous hepatitis (Fig. 2) were seen in 66.7% (6/9) cases, followed by granulomatous splenitis (Fig. 3) in 44.44% (4/9) of cases, and granulomatous myositis in 33.3% (3/9) of the cases.

A female rhesus monkey (NHP 2) with no pulmonary involvement showed many granulomas in the trachea, lymph nodes, mesentery, duodenum, pancreas, liver and spleen. In addition to the mesenteric granulomas, this rhesus female presents multifocal variable-sized dark-red cystic masses extending from the uterus to the abdominal wall and serosa of multiple intestine segments, which contained a large amount of dark-red fluid on the cut section. These dark-red cystic masses also are found in multiple abdominal surface organs of a cynomolgus monkey (NHP 9) with considerable enlargement of the mediastinal lymph node and diffuse replacement of tissue architecture by amorphous white areas, multifocal to coalescent yellowish granulomas.

About the New World Monkeys (NWM), the typical pulmonary granulomas were seen in 16.7% (1/6) of the cases, in the Uta Hick's Bearded Saki. The main gross change observed in the NWM was moderate lung increase volume with marked edema in 83.3% (5/6) of the cases, followed by different degrees and severity of pulmonary consolidation with irregular multifocal red-dark areas 66.7% (4/6) of the cases. In two marmosets (NHPs 10 and 11) has been noted evidence of the hepatic lobular pattern with an increase of the lumen diameter of biliary ducts and gallbladder.

Granulomatous changes were seen in 50% (3/6) of the NWM cases. A marmoset (NHP 12) presented granulomatous myositis, periostitis, and pleuritis (Fig. 5). The squirrel monkey (NHP 14) showed granulomatous hepatitis, with many granulomas in the liver parenchyma. In one case, multisystemic granulomatous change was observed in the Uta Hick's Bearded Saki (NHP 15). In this case, the pulmonary granulomas were multifocal and well-delimited structures, sometimes encapsulated, with different sizes in all pulmonary lobes. Some areas of pulmonary lobes creaked when cut (Fig. 6). Furthermore, this nonhuman primate presents extrapulmonary spread into the lymph nodes, liver (Fig. 7), spleen and kidney (Fig. 8).

2.4 Histopathological and immunohistochemical findings

In this study, 66.7% (6/9) of Old World Monkeys (OWM) and 83.3% (5/6) of New World Monkeys (NWM) showed pulmonary changes at histology. A severe multifocal to coalescent granulomatous pneumonia was seen in all six rhesus monkeys with pulmonary changes and 16.7% (1/6) of NWM tuberculosis cases. In 66.7% (4/6) of NWM tuberculosis cases, different degrees of diffuse foamy interstitial pneumonia were observed with no evidence of necrosis or pulmonary granuloma formation. The pulmonary changes of all nonhuman primates with tuberculosis are summarized in Table 3.

Placeholder for Table 3; see Supplementary Files section.

At histology, lung evaluation of the OWM revealed multiple areas with typical caseous granulomas, solid-cellular granuloma (non-necrotizing), and suppurative granuloma in each of the six rhesus monkeys. The caseous granulomas present an acellular center with a large amount of amorphous hypereosinophilic material and pyknotic debris surrounded mainly by epithelioid macrophages, multinucleated giant cells (Langhans and Foreign body giant cells), and a variable number of lymphocytes, neutrophils and rarely foam cells. In the periphery of the caseous granulomas, often were observed solid-cellular granulomas composed of epithelioid macrophages and multinucleated giant cells. In 66.7% (4/6) of cases was observed variable bronchial epithelium ulceration and neutrophils infiltration (NHPs 1, 4, 5, and 7) with the formation of multiple cavities into the major airways (Fig. 9).

Besides the lungs, caseous granulomas and solid-cellular granulomas were also found in different severities within multiple organs of these rhesus monkeys. In NHPs 1, 3, 4, 5, 6, and 7 a severe replacement of the lymph node architecture by numerous caseous and solid-cellular granulomas was observed. The liver (Fig. 10) (NHPs 1, 3, 4, 5, and 7), spleen (NHPs 3,4,5, and 7), pleura (NHPs 1, 3, 5 and 7), skeletal muscle (NHPs 4, 5 and 6), esophagus (NHP 1), stomach (NHP 4), pancreas (NHP 4),

duodenum (NHP 4), kidney (NHP 1), adrenal (NHP 1), joints (NHP 6), brain (NHP 1), and nerves (NHP 6) also presented multiple granulomas in different severities.

In 50% (3/6) of OWM cases with granulomatous pneumonia, it was possible to visualize straight or slightly curved bacilli, with 1 to 5 μm long and 0.2 to 0.8 μm wide into the areas of necrosis or inside the cytoplasm of macrophages, epithelioid macrophages and multinucleated giant cells using the Ziehl-Neelsen technique (Fig. 11). Immunohistochemistry with anti-*Mycobacterium tuberculosis* showed intact and degenerated bacilli amidst the necrotic areas and intracytoplasmic in multinucleated giant cells, macrophages and epithelioid macrophages, all six cases of granulomatous pneumonia analyzed (Fig. 12).

A rhesus monkey (NHP 2) with no pulmonary changes showed multifocal to coalescent typical caseous granulomas in the trachea, lymph nodes, mesentery, duodenum, pancreas, liver and spleen. The dark-red cystic masses of the abdomen wall (Fig. 13) and mesenteric nodules at the histological evaluation, in addition to the tuberculosis granulomas, a moderately cellular proliferation composed of columnar and ciliated endometrial cells with a moderate amount of clear eosinophilic cytoplasm and round nuclei. Multifocally, there are areas with a moderate amount of free red blood cells (hemorrhage), accompanied by macrophages with abundant intracytoplasmic brown pigments (hemosiderophages). These findings are compatible with endometriosis (Fig. 14).

A cynomolgus monkey (NHP 9) without any pulmonary impairment also presented endometriosis. Moreover, this cynomolgus presents a multifocal to coalescent granulomatous lymphadenitis with intense replacement of the typical architecture of the lymph node by severe caseous granulomas formation. In a rhesus monkey (NHP 8), histological changes were observed just in the cardiac ganglia. The neuronal cell bodies of the ganglia are expanded by a few multinucleated giant cells of foreign body type. Adjacent to that areas and expanding the epicardium were seen well-demarcated multifocal areas with necrotic center and dystrophic mineralization.

Out of three New World Monkeys that present histopathological pulmonary changes, two (66.7%) marmosets and one squirrel monkey (NHP 13) presented different degrees of the proliferation of foamy macrophages that thicken the alveolar septa and infiltrate the air space (Fig. 15). These macrophages exhibit ample foamy cytoplasm and often concentric and lateralized nucleus. Although few giant cells were seen in one marmoset (NHP 10), no evidence of necrosis or granuloma formation was noted in lung sections or any other organ on this NWM.

A squirrel monkey (NHP 14) showed multifocal granulomatous hepatitis with a large number of solid-cellular granulomas and few caseous granulomas surrounded mainly by some epithelioid macrophages, multinucleated giant cells, and lymphocytes. The marmoset (NHP 12) presents a granulomatous pleuritis with a large caseous granuloma in the intercostal muscle adjacent to the pleura. A mild diffuse interstitial pneumonia with the predominance of lymphocytes was observed in this marmoset. That was the only case with pneumonia in this group.

A significant number of lymphocytes and eosinophils are seen around the ectatic ducts, and several fibroblastic proliferation and multifocal areas increased in the number of bile ducts (biliary hyperplasia). Two marmosets of the early activation stage group (NHPs 10 and 11) showed a severe lymphocytic and eosinophilic proliferative cholangitis at histology, with multiple free eggs and structures compatible with adult trematodes inside and obstructing partially the lumen of biliary ducts, that were often ecstatic. These adult trematodes have prominent oral and ventral suckers, a thin outer integument covering a somatic musculature, a parenchymatous matrix, a digestive tract with paired ceca, vitellaria, and a uterus with numerous, oval, yellow-brown singly operculated, thick eggs with miracidia.

In the only case of granulomatous multifocal to coalescent bronchopneumonia in the NWM, the Uta Hick's Bearded Saki showed a large number of well-demarcated and encapsulated mineralized granulomas with variable sizes in the pulmonary parenchyma (Fig. 16). These granulomas are often surrounded by a significant number of foamy macrophages with abundant cytoplasm, epithelioid macrophages, lymphocytes and a few multinucleated giant cells and neutrophils. Adjacent to those areas, the alveolar space showed a severe amount of amorphous eosinophilic material (edema). Sometimes, multifocal fibroblastic proliferation in the alveolar wall is seen in the lung sections. A great number of foamy macrophages infiltrated multifocally, the airways and the bronchial and bronchiolar lumen.

Through the evaluation using the Ziehl-Neelsen technique, a few bacilli, predominantly intracellular in foamy cells of the marmosets and extracellularly in the necrosis areas of the Uta Hick's Bearded Saki (Fig. 17), were visualized in 83.3% (5/6) of the cases. There was evidence of intracellular bacilli in all cases, mainly in the airways and intrabronchial foamy macrophages (Fig. 18). The Ziehl-Neelsen and immunohistochemical results of each case of NHP with tuberculosis are available in Table 4.

Placeholder for Table 4; see Supplementary Files section.

2.5 Molecular findings

The molecular analysis was performed in fourteen of fifteen cases of the nonhuman primates in this study. In the molecular detection with oligonucleotides INS1 and INS2, 64.28%(9/14) were positive, while with oligonucleotides TB11 and TB12, 14.28%(2/14) were positive and submitted to sequencing, confirming the diagnosis for CMTB. The GeneXpert® system was able to detect the presence of tuberculosis complex species DNA in all analyzed samples. Additionally, all samples were identified as sensitive to the antibiotic rifampicin, amplifying the genes *rpoB1*, *rpoB2*, *rpoB3*, and *rpoB4*. The molecular results of each case are available in Table 4.

2.6 Bacterial culture

The bacterial culture was performed in six of fifteen cases of the nonhuman primates in this study. In all cases was observed the growth of *Mycobacterium tuberculosis* complex bacteria. The bacterial culture results of each case are available in Table 4.

2.7 Tuberculosis stage classification

Based on clinical, morphological, immunohistochemical, and, when possible molecular and bacteriological results, the nonhuman primates are classified in different tuberculosis stages (Table 5). Out of nine Old World Monkeys (OWM), 66.7% six cases were classified as active-chronic tuberculosis stage. Among these primates, typical pulmonary granulomas were seen in all cases in this group and clinical respiratory signs in 83.3% (5/6) of cases. In 33.3% (3/9) OWM cases, the stage was classified as extrapulmonary due to the absence of gross or histologic granulomatous pneumonia. Extrapulmonary tuberculosis was seen in two cynomolgus monkeys (*M. fascicularis*) and one rhesus monkey (*M. mulatta*). In this group, respiratory signs were observed in 33.3% (1/3) of the cases.

Placeholder for Table 5; see Supplementary Files section.

In the New World Monkeys (NWM), 50% (3/6) of the cases were classified as the early-activation stage, 33.3% (2/6) as extrapulmonary stage and 16.7% (1/6) as latent-reactivation tuberculosis stage. In the early-activate group, 66.7% (2/3) of the primates were marmosets (*Callithrix* sp.), and 33.3% (1/3) were squirrel monkeys (*S. ustus*). In none of the early activation cases, pulmonary granulomas were observed, and only the squirrel monkey showed clinical respiratory signs. The extrapulmonary stage group was composed of one marmoset (*Callithrix* sp.) and one squirrel monkey (*S. ustus*). No clinical respiratory signs were observed in the NWM of the early-activate stage. The latent-reactivation stage was observed in the Uta Hick's Bearded Saki (*C. utahickae*). This primate had typical latent pulmonary granulomas, and no clinical respiratory signs were observed.

3. Discussion

Prior studies with experimental infections of *Mycobacterium tuberculosis* Complex (MTBC) in nonhuman primates (NHPs) have described the morphological heterogeneity of tuberculosis (Capuano et al., 2003; Lin et al., 2014; Cadena et al., 2017). In humans, pulmonary granuloma formation is considered the pathological hallmark of tuberculosis (Wadee and Wadee, 2021). In NHPs infected with MTBC, a large spectrum of lesions can be seen within and between monkeys of the same stage classification as well described in humans (Capuano et al, 2003; Lin et al, 2009; Flynn and Klein 2011). Although the typical tuberculosis presentation was observed in some cases, the absence of pulmonary granuloma did not exclude the tuberculosis occurrence of extrapulmonary impairment or early pulmonary changes in NHPs of the Old and New World naturally infected with MTBC. We believe that the main differences between the tuberculosis pathology in NHPs are due to the stage disease presentation.

We observed predominance of the chronic-active tuberculosis stage in OWM, especially in rhesus monkeys (*Macaca mulatta*), compared to the cynomolgus monkey (*M. fascicularis*). Presumably, this fact occurs because the rhesus macaques are more susceptible to the *M. tuberculosis* infection than cynomolgus macaques (Langermans et al., 2001; Maiello et al., 2018). This fact may have favored the activation stage of the disease in rhesus monkeys. In this natural occurrence of chronic-active

tuberculosis in rhesus monkeys, all nonhuman primates present changes compatible with descriptions of experimental infectious tuberculosis in this species (Zhang et al., 2011; Zhang et al., 2014). In our study, all rhesus monkeys of chronic-active tuberculosis present a full spectrum of histopathological changes with different granulomas types in the same individual, as well seen in humans (Capuano et al., 2003).

Extrapulmonary tuberculosis refers to TB involving potentially any organ other than the lungs, and reports of this presentation in macaques are scarce in the literature (Stockinger et al., 2011; Lee 2015). According to Capuano et al., (2003), the extrapulmonary spread was variable among nonhuman primates, and when the disease is advanced, the nonhuman primates can exhibit extrapulmonary dissemination. The macroscopic and histopathological granulomatous lesions in several organs of a *M. mulatta*, lymph nodes and heart of *M. Fascicularis*, liver of the *S. ustus*, skeletal muscle and parietal pleura of *Callithrix* sp. show the plasticity of MTBC to cause different changes in multiple organs in infectious individuals even when pulmonary changes were absent. Due to these findings, we suspect that the systemic involvement has resulted from the hematogenous spreading of the MTBC bacilli after the initial infection.

Hematogenous dissemination within organs can occur in tuberculosis infectious. Despite wide dissemination of *Mtb* during primary infection, the majority of infected, but otherwise healthy, individuals resolve these lesions without becoming symptomatic (Sakamoto, 2012). Nonhuman primates exposure to *M. tuberculosis* results in a wide range of outcomes (Scanga and Flynn, 2014). Unlike the granulomatous changes in many organs, our extrapulmonary results suggest a possible pulmonary resolution due to an absence of clinical respiratory signs in the mostly nonhuman primates with this stage presentation. None factor was associated as a cause of the high prevalence of extrapulmonary tuberculosis in nonhuman primates of this study. The concomitance of endometriosis and abdominoperitoneal tuberculosis were described in humans, especially in infertile women (Sharma et al., 2017). These condition can co-exist in nonhuman primates without previously described mechanism. We suggest further studies to determine the pathogenesis of concomitance.

The formation of foam cells is a manifestation of maladaptive responses occurring during many inflammatory conditions (Hotamisligil, 2017). In humans, there is a wide literature detailing the presence of foamy macrophages in tuberculosis granulomas (Hunter et al., 2007; Peyron et al., 2008). Although the occurrence of foam cells is typically associated with necrotic granulomas (Peyron et al., 2008), the absence of necrosis, granulomatous changes or interstitial fibrosis in the early-activation tuberculosis stage can indicate that foamy macrophages seemed to be a key component in the initial activation pathogenesis of tuberculosis in nonhuman primates.

The lesions of early pulmonary tuberculosis can develop along several pathways (Hunter, 2011). In the early-activation stage, we observed diffuse interstitial foamy pneumonia. Although lipidic pneumonia was associated with post-primary tuberculosis (Hunter et al., 2007), no evidence of endobronchial tuberculosis producing bronchial obstruction, necrosis, or cavitation was found in the NHPs lungs with early-activation stage in this study. In one marmoset of the early-activation lung stage, we observed a great number of interstitial foamy macrophages and few multinucleated giant cells without necrosis.

That can be an initial fact associated with tuberculosis granuloma formation. Presumably, the initial granuloma formation mechanism, especially in NWM needs to be better elucidated in further studies.

Studies have proposed that intracellular macrophage bacilli overproduce Mtb lipids; these lipids consolidate in the internal vesicles in the multivesicular body and are subsequently exocytosed into the extracellular medium (Beatty et al., 2000; Beatty et al., 2001). It was demonstrated that foam cell formation is induced explicitly by oxygenated forms of mycolic acid, such as oxygenated ketomycolic and hydroxyl-mycolic acids synthesized by pathogenic mycobacterial species such *Mtb* (Peyron et al., 2008; Russel et al., 2009). In our study, the presence of bacilli in the foamy macrophages was confirmed through de acid-fast stain and immunohistochemistry technique.

As in humans, tuberculosis in nonhuman primates can be stably maintained, spontaneously reactivate, or reactivate in response to some immune suppression (Lin et al., 2009; Lin and Flynn, 2010). Did not possible to determine an immunosuppression factor associated with the latent-reactivation stage in Uta Hick's Bearded Saki in this study, and that appears to have spontaneously reactivated a latent infection. We observed an intense intracytoplasmatic immunolabelling to anti-*Mycobacterium tuberculosis* on foamy macrophages of the latent-reactivation stage. This fact also indicates the foam cells formation due to an active *M. tuberculosis* replication. Besides the necrotizing granulomas, this monkey showed nonnecrotizing granulomas in the lung tissues that have not been observed in latent infection in nonhuman primates (Lin et al., 2009).

This study shows that clinical respiratory signs should not be considered solely for the clinical suspicion of tuberculosis in nonhuman primates. Coughing is infrequent in nonhuman primates with tuberculosis but may occur (Mansfield and Fox, 2019), like in the three cases of rhesus monkey and one squirrel monkey (*S. ustus*) from this survey. In our study, weight loss, lethargy and anorexia were the most frequent clinical signs in nonhuman primates with tuberculosis in according to described by Simmons and Gibson (2012) and Via et al. (2003).

The tuberculosis diagnosis in the nonhuman primates in this study was made based on the association of pathological, immunohistochemical, molecular, and when possible bacteriological culture findings. The presence of mycobacterial antigens and tissue morphology can be evaluated together using the immunohistochemistry technique (Karimi et al., 2014). Due to the high sensitivity of this technique compared to the acid-fast stain, we recommend for routine diagnostic its use even in cases without typical tuberculosis microscopic changes. The molecular analysis presents high sensitivity and specificity for the discrimination of microorganisms belonging to the *M. tuberculosis* Complex (Neshani et al., 2018). Although ampicillin resistance was not diagnosed in any case in the present study, we suggest using GeneXpert® as a surveillance tool for bacterial resistance to antibiotics.

All nonhuman primates of this study were naturally infected with *Mycobacterium tuberculosis* Complex, although reports stated that the Old World Monkeys are more susceptible than New World Monkeys (Brack, 1987; Montali, 2001; Blanchard and Russell-Lodrigue 2012). No predisposing factors were observed in Old World nonhuman primates compared to New World primates. The occurrence of TB in

free-ranging nonhuman primates is considered uncommon (Rocha et al, 2011). We believe that all nonhuman primates under human care with direct contact with humans are susceptible to *M. tuberculosis* infection. Due to the natural occurrence of MTBC infection in this study was not possible to determine the route, strain, infectious dose and time of evolution of the tuberculosis infection.

Complete and specific exams for the *antemortem* tuberculosis diagnosis must be carried out constantly in the nonhuman primates colonies, even when clinical respiratory signs are not evident. Due to the possible retransmission from monkeys to man (Mätz-Rensing et al, 2015), the occurrence of tuberculosis in nonhuman primates offers a potential health risk for other staff members. Owing to the inherent risk a nonhuman primate infected with tuberculosis poses to the colony and staff, it is recommended that monkeys infected with or suspected of being infected with tuberculosis be euthanized (National Research Council, 2003; Bushmitz et al., 2009).

In conclusion, we observe a wide spectrum of morphological features comparing the nonhuman primates diagnosed with different tuberculosis stages. Classical granulomatous pneumonia was observed in the chronic-active and latent-reactivation stages but not in the extrapulmonary and early-activation stages. The early-activation stage in primates is characterized by foamy interstitial pneumonia without typical tuberculosis granulomas. Due to this fact, tuberculosis should be included as a cause of foamy interstitial pneumonia in nonhuman primates, especially in the New World species. Experimental studies need to be realized to elucidate the exact role of the foamy macrophage in the early stage of tuberculosis. We recommend the addition of immunohistochemistry as a diagnostic tool of tuberculosis even when typical macroscopic or histologic changes are not observed.

Declarations

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Tables

Tables 1 - 5 can be viewed/downloaded from the Supplementary Files section.

Figures

Figure 1

Gross findings of rhesus monkey (*Macaca mulatta*) with chronic-active tuberculosis stage. Figure 1. Multifocal to coalescent granulomatous pneumonia. with well-demarcated structures, sometimes elevated, firm yellow-white or grayish nodules of different sizes. The lungs are diffusely firm, enlarged and reddish with multiple consolidations areas. Inset: multiple cavitation areas of different sizes on the airways

Figure 2

Gross findings of rhesus monkey (*Macaca mulatta*) with chronic-active tuberculosis stage. Figure 2. Multifocal granulomatous hepatitis. Inset: hepatic cut surface with multifocal granulomas of variable size on parenchyma

Figure 3

Gross findings of rhesus monkey (*Macaca mulatta*) with chronic-active tuberculosis stage. Figure 3. Multifocal granulomatous splenitis.

Figure 4

Gross findings of rhesus monkey (*Macaca mulatta*) with chronic-active tuberculosis stage. Figure 4. Multifocal granulomatous neuritis. There is a great number of granulomas adhered on the nerves sheets.

Figure 5

Gross findings of New World Monkeys with different tuberculosis stages. Figure 5. Under the parietal pleura and skeletal muscle, multifocal areas of granulomas formation in a *Callithrix* sp. with extrapulmonary tuberculosis.

Figure 6

Gross findings of New World Monkeys with different tuberculosis stages. Figure 6. Lung cut surface of an Uta Hick's Bearded Saki with latent-reactivation tuberculosis stage. Note the multifocal areas with well demarked yellow-wish areas n the airways.

Figure 7

Gross findings of New World Monkeys with different tuberculosis stages. Figure 7. Systemic granulomatous spread of an Uta Hick's Bearded Saki with latent-reactivation tuberculosis stage. Note granulomatous hepatitis and mesenteric granulomatous lymphadenitis.

Figure 8

Gross findings of New World Monkeys with different tuberculosis stages. Figure 8. Multifocal granulomatous nephritis of an Uta Hick's Bearded Saki with latent-reactivation tuberculosis stage.

Figure 9

Microscopic findings of Rhesus Monkey (*Macaca mulatta*) with chronic-active tuberculosis stage. Figure 9. Granulomatous severe pneumonia. The airways are replaced by epithelioid macrophages, multinucleated giant cells (Langhans and Foreign body giant cells), a variable number of lymphocytes, neutrophils and rarely foam cells. There is a focal area of ulceration of bronchial epithelial cells and cavitation to the airways (arrow). There are multifocal areas of necrosis with a great number of pyknotic debris and hyperosinophilic amorphous material. (HE, Obj 2,5x).

Figure 10

Microscopic findings of Rhesus Monkey (*Macaca mulatta*) with chronic-active tuberculosis stage. Figure 10. Granulomatous hepatitis with a central large caseous granuloma. (HE, Obj. 5x).

Figure 11

Microscopic findings of Rhesus Monkey (*Macaca mulatta*) with chronic-active tuberculosis stage. Figure 11. Few acid-fast bacilli into the cytoplasm of giant cell multinucleated (arrow) and in the extracellular medium. (Ziehl-Neelsen, Obj. 40x).

Figure 12

Microscopic findings of Rhesus Monkey (*Macaca mulatta*) with chronic-active tuberculosis stage. Figure 12. Intense intracytoplasmic immunolabeling anti-*Mycobacterium tuberculosis* in the giant cells multinucleated with evidence of intact and degenerated bacilli in the cytoplasm. Besides, note the great number of bacilli into the extracellular medium (IHC anti-*Mycobacterium tuberculosis*, Obj. 40x).

Figure 13

Pathological findings of Rhesus Monkey (*Macaca mulatta*) with concomitant extrapulmonary tuberculosis and endometriosis. Figure 13. Gross finding of abdominal wall with granulomatous white nodules (arrow) and dark-red cystic masses (arrowhead).

Figure 14

Pathological findings of Rhesus Monkey (*Macaca mulatta*) with concomitant extrapulmonary tuberculosis and endometriosis. Figure 14. Concomitance of endometrial cystic proliferation and a caseous granuloma (asterisk) (HE, Obj. 2,5x). Inset: moderately cellular proliferation composed of columnar and ciliated endometrial cells with a moderate amount of eosinophilic cytoplasm and round nuclei. (HE, Obj. 40x).

Figure 15

Microscopic findings of New World Monkeys with different tuberculosis stages. Figure 15. Interstitial foamy pneumonia in a *Callithrix* sp. with early-activation tuberculosis. The septa are distended by a great number of foamy macrophages and lymphocytes. (HE, Obj 40x). Inset: Intense intracytoplasmic immunolabeling anti-*Mycobacterium tuberculosis* in foamy macrophages with evidence of intact and degenerated bacilli in the cytoplasm. (IHC anti-*Mycobacterium tuberculosis*, Obj. 40x).

Figure 16

Microscopic findings of New World Monkeys with different tuberculosis stages. Figure 16. Multifocal solid-cellular and mineralized granulomatous with some areas of caseous necrosis in the lung of Uta Hick's Bearded Saki (*Chiropotes utahickae*) with latent-reactivation tuberculosis stage (HE, Obj. 2,5x).

Figure 17

Microscopic findings of New World Monkeys with different tuberculosis stages. Figure 17. Acid-fast bacilli into the extracellular medium (arrow) of a caseous lung granuloma of an Uta Hick's Bearded Saki (*Chiropotes utahickae*) with latent-reactivation tuberculosis stage. (Ziehl-Neelsen, Obj. 40x).

Figure 18

Microscopic findings of New World Monkeys with different tuberculosis stages. Figure 18. Intense intracytoplasmic immunolabeling anti-*Mycobacterium tuberculosis* in the intrabronchial foamy macrophages with evidence of intact and degenerated bacilli in the cytoplasm. Besides, note the great number of bacilli into the intra and extracellular medium at the submucosa of bronchi (IHC anti-*Mycobacterium tuberculosis*, Obj. 40x).

Supplementary Files

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- [Tables1through5.pdf](#)