Status of the *Mycobacterium bovis* and the interrelationship with human health in Amazonas State, Brazil

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Background

Tuberculosis (TB) remains one of the world's major health problems for both humans and animals. In 2016, an estimated 10.4 million people developed TB and 1.8 million died from the disease, 400,000 of whom were HIV-positive [1]. Brazil ranks 18th in tuberculosis (TB) burden, accounting for 0.9% of cases worldwide. Brazilian national mortality and incidence rates were reduced by 38.9% (3.6 to 2.2 per 100,000 persons) and 34.1% (51.8 to 33.2 per 100,000 persons), respectively, from 1990 to 2015 [2]. In contrast to the national figures, in the Amazonas State the TB incidence rate have been rising in the last decade, reaching 67.2 cases per 100,000 persons in 2016 - the highest incidence in the country, third highest mortality rate due to TB (3.2/100.00) [2]. Thus, despite the efforts of the local Amazonas authorities in diagnosing TB; and free access to treatment of TB, a significant reduction in TB cases has not been achieved.

Intriguingly, the common sense epidemiological frame of TB component causes – exposure to the Mycobacterium, Low Social Economic Status, and High Population Density, which do not reasonably explain neither the rate nor the trend of the TB incidence in Amazonas State.

Mycobacterium bovis (*M. bovis*), the causative agent of bovine tuberculosis (bTB) and M*ycobacterium tuberculosis* are related organisms of the *M. tuberculosis* complex. *Mycobacterium tuberculosis* is the most common cause of human TB, but an unknown proportion is due to *M. bovis*. In fact, human disease with *M. bovis* is well described and historically was a common cause of TB-transmitted by infected dairy products [3]. In industrialized countries, animal TB control and elimination programs, together with milk pasteurization, have drastically reduced the incidence of disease caused by *M. bovis* in both cattle and humans [4]. In developing countries, however, animal TB is widely distributed, control measures are not applied or are applied sporadically, and pasteurization is rarely practiced [5].

Alarmingly, the major risk factors associated with zoonotic TB (due to *M. bovis*), such as occurrence of *M. bovis*, low rates of milk pasteurization, and high consumption of products from raw milk are routine in Amazonas's municipalities, and physicians might be unaware of the risk of the occurrence of zoonotic infection, and therefore specific culture for *M. bovis* is not requested.

In 1989, a phenotypical survey on pulmonary secretions from 516 TB patients living in the state, and found 8 Mycobacteria other than *M. tuberculosis*, 7 of them considered potentially pathogenic [6]. Since then, no other studies were performed in order to profile the TB strains occurring in Amazonas State nor even trying to identify multibacillary infections.

Objectives and Hypotheses

Our overarching hypothesis is that the contribution of *M. bovis* to human tuberculosis is underestimated in Amazonas State and may explain the much higher incidence and prevalence of human TB in Amazonas State than the rest of Brazil.

To test our overarching hypothesis three specific aims conducted, each with its specific hypothesis.

I. Determine the epidemiological profile of *M. bovis* infection in samples of TB suspected animals from slaughterhouse in Manaus micro region;

We hypothesize that at least 13%, of the samples from suspected animals collected in slaughterhouses in Manaus, will be infected with the *M. bovis*.

II. Assess of the degree of contamination by *M. bovis* in milk and dairy products produced in Manaus micro region,

We hypothesize that at least 10% of the milk and cheese from raw milk produced in the infected farms will be infected by *M. bovis*.

III. Provide evidence of the occurrence of *M. bovis* infection in humans in Amazonas State;

We hypothesize that at least 2%, of the samples from TB patients collected at the regional reference laboratory in Manaus – AM, will be infected with the *M. bovis*.

Significance

The proposal represents the first "One Health" approach of the tuberculosis problem in the Amazon region, looking for links between the health of humans and the health of animals. This project has the potential to clarify key questions about the epidemiology of the TB in Amazonas State, such as the identification of the *M. tuberculosis* strains present in the region, the possibility of zoonotic infection, the chain of infection, and regional risk factors. Altogether, this will allow not only the improvement of the diagnosis, treatment, and the control of the disease in Amazonas State, but also, the research will represent an opportunity to seed the integration between human and veterinary medicine, in actions to monitor and control public health threats.

Ultimately, the answers obtained on this project will allow the improvement of the public health policies and the lowering of the TB incidence rates in the region

Materials and Methods

Study design

This project is a cross-sectional study, based on data provided by animal health agencies and primary data generated from surveys to be conducted in slaughterhouses, farms, and local laboratories of TB diagnosis at Manaus micro-region. The primary data of human population will be obtained from patients suspected of TB at Amazonas State regional laboratory.

Duration of study

The estimated study duration will be 36 months, from January 2017 to December 2019.

The cross sectional study shall cover slaughterhouses during the period from January to December 2017.

The samples, collected at the farms and the reference laboratory, will be processed from January to December 2018.

Permission to conduct human and animal scientific research

The project has all necessary approvals from MSU-IRB and IACUC, and will obtain approvals from IFAM's CEPSH and CEUA.

Determining the epidemiological profile of *M. bovis* infection in samples of TB suspected animals from slaughterhouse in Manaus micro-region (Designed to test hypothesis 1).

Due to challenging logistics and costs, this study will be restricted to the Manaus micro-region, which represents the general characteristics of the herds in Amazonas municipalities. Official veterinarians at the two slaughter houses will collect at least 175 samples from carcasses of animals (cattle or buffalo) with lesions suspected of TB. The collection and processing of specimens for culture will be performed following the 2012 OIE Terrestrial Manual [7]. The samples will be divided into two parts (one for culturing and the other for DNA isolation), stored, and transported on ice to a biosafety laboratory. After culturing and isolating has been carried out, the material will be subjected to molecular diagnostic techniques Tríplex-PCR, 24-loci MIRU-VNTR, and Whole Genome Sequence to detect distinct *Mycobacterium bovis* profiles.

The microbiological and molecular analysis will be carried out at the Laboratory of Immunology of the Beef Cattle National Center in Campo Grande, Mato Grosso do Sul State, in Brazil.

Case definition: An animal will be considered positive for TB by *M. bovis*, when at least a single tissue sample exhibited amplification in the PCR or exhibit Acid Fast Bacilli cultures confirmed by the PCR.

Statistical analysis of expected data:

Type of Outcome: Count data to be generated to compute *M. bovis* prevalence.

Risk factors: From each carcass sampled epidemiological information such as: origin, sex, age, specie (cattle or buffalo), type of farming and husbandry, herd history of tuberculosis and sanitary practices that can be associated with the risk of infection by the disease, will be obtained from the GTA (Animal Transport Guide).

Given the nature of the outcome and number of risk factors a multivariable logistic regression model will be used to assess the influence of the aforementioned risk factors on the prevalence, using 95% confidence intervals (P ≤ 0.05).

Assessment of the degree of contamination by *M. bovis* in milk and cheese produced in Manaus micro-region (Designed to test hypothesis 2).

In order to evaluate the distribution of *M. bovis* in milk and cheese from the positive farms, isolation and tests will be performed to ascertain the presence of the Mycobacterium. The identification will be performed by classical biochemical tests and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis.

The sampling of raw milk will be collected from the milk can. Two samples of 15 ml each will be collected. In the farms where cheese are available, 250g will be sampled by taking thin cuts of the cheese surface. The raw milk and cheese samples will be transported from the farms on ice and then frozen until the analysis. DNA PCR-based method reported by Quigley, et al, 2012 will be used [8].

The microbiological analysis will be performed at the Regional reference laboratory (LACEN) in Manaus. The molecular analysis will be carried out in the Laboratory of Immunology at the Beef Cattle National Research Center in Campo Grande, Mato Grosso do Sul State, in Brazil.

Case Definition: Confirmation of *M. tuberculosis* complex isolates as *M. bovis* will be accomplished using a PCR-based typing method targeting the *M. tuberculosis* complex chromosomal region-of-difference deletion loci, as described previously.

Statistical analysis of expected data:

Outcome: Count data to be generated to compute *M. bovis* prevalence.

Risk factors: Origin by municipalities and type of cheese.

The unit of analysis will be the individual sample. Given the nature of the outcome and number of risk factors a multivariable logistic regression model will be used to analysis the data to be obtained.

To provide evidence of the occurrence of *M. bovis* infection in humans in Amazonas State (Designed to test hypothesis 3).

In order to determine the influence of the *M. bovis* in the incidence of human tuberculosis in Amazonas State, the tuberculin skin test, bacteriological and molecular investigations will be conducted on human populations that will cover the farmers and dairy workers from the positive farms and patients suspected of TB cases routinely analyzed at the laboratory in the municipalities of Autazes and Careiro da Várzea.

Reference Population:

• Respiratory symptoms (individuals with cough for more than two weeks) in the two selected municipalities (Autazes and Careiro da Várzea).

• Human contacts (workers / inhabitants) of sites with positive results for *M. bovis* in animals (cattle).

At the positive farms a convenience sampling will be performed with the dairy workers and farmers. The sample size will be estimated in approximately 756 subjects, at Careiro da Várzea, 572 samples will be collected based on the same indicators, making a total of at least 1,730 human samples.

The standard diagnosis will be performed as prescribed by the Brazilian National Program, however, culture techniques will be conducted using an approach that is suitable for recovering both *M. tuberculosis* and *M. bovis*. The three step approach molecular diagnosis will be performed as described previously.

All procedures related to the determination of the occurrence of zoonotic TB will be conducted in partnership with the Health Surveillance Foundation (FVS) of the Amazonas State. The microbiological analysis will be performed at the Regional reference laboratory (LACEN) in Manaus. The molecular analysis will be carried out in the Laboratory of Immunology at the Beef Cattle National Research Center in Campo Grande, Mato Grosso do Sul, in Brazil.

Statistical analysis of expected data:

Outcome: Prevalence (count data).

Risk factors: Age, consumption of unpasteurized milk, consumption of unpasteurized dairy products, contact with infected animals, malnutrition, rural or urban origin, overcrowding, occupation, latent or active infection, TB resistant condition, family history of TB, and HIV status prior treatment history.

The unit of analysis will be the subject. Given the nature of the outcome and number of risk factors, a multivariable logistic regression model will be used to analysis the data to be obtained.

Preliminary results

To date 946 animals were inspected and 63 samples from suspected animals were collected, 27 farms from 13 municipalities were involved. Twenty nine samples had growing compatible with *M. bovis* and twenty six animals had positive results for *M. bovis* confirmed by the PCR analysis. Considering only samples positive in the microbiological and molecular analysis the sampling prevalence to date reach 41.26% in samples from slaughterhouses. Seven municipalities presented infected animals, the sampling prevalence were Apuí and Autazes (100%), Manacapuru (75%), Urucará (66%), Careiro da Varzea (60%), Itacoatiara, and Parintins (33%). The next steps of the project are: to trace the farms with positive animals to assess the contamination by M. bovis in milk and cheese, perform a screening in those farms and two municipalities in order to determine the presence of zoonotic tuberculosis in Amazonas State. The screening will be performed together with an epidemiological investigation aiming to uncover the possible risk factors for the disease transmission.

Conclusions

To date the results show that the disease is widespread in the State. In addition the prevalence is much higher than predicted (41.26% vs 13%), indicates that the bovine tuberculosis is a significant problem in Amazonas State.

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