Mycobacterial isolation from *Felis concolor* in captivity

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Abstract

This study was made in a wildlife preserve from Argentina where a previous tuberculosis report in *Felis concolor* has been done. The aim was to identify mycobacterial species isolated from the orpharynx of five American lions using bacteriological and molecular biology techniques on cases with nonspecific clinical signals. Samples were collected after sedation. They were treated in order to isolate *Mycobacterium*. Bacteriological differentiation was made using biochemical tests. Polymerase chain reaction has been performed to detect *hsp65,* IS*6110* and IS*1081*. Acid fast bacilli were present in four specimens and from them were isolated slowly growing mycobacteria. The strains were differentiated as *M. gordonae* in two cases and *M. simiae*, *M. scrofulaceum* and *M. avium/ intracellulare* in one case each other. The strains were identified as *M. gordonae* in three cases and *M. avium* III or *M. simiae* in two by PRA. The role of feral cats in the epidemiology of nontuberculous mycobacterial diseases remains to be further investigated.

Key words:
Felis concolr.
Nontuberculous mycobacteria.
Zoological garden.

Introduction

American lion is a carnivore mammal spread out American land, from Canadian southern territory to austral Patagonia. Its detailed taxonomic classification is Class: Mammalia, Infraclass: Eutheria, Order: Carnivora, Family: Felidae, Genus: *Felis*, Specie: *concolor*. It is 120 cm long, 65 cm height and its tail is 60 cm long, because of these sizes it is the biggest carnivore in America land occupying the top of the food chain. As happens with other felines its staple diet is adapted to the habitat. To obtain their preys American lions mimic the environment and chase small and medium size animals (monkeys, ratites, sudamerican camelids, birds and other warm-blooded animals), it also feeds itself on fishes and small lizards.[1]

As population grows wildlife environment has been bordered on places with no fully access so that when food supplies are not enough *Felis concolor* chases in bovine, ovine and camelids herds causing important economic losses. In some areas it is almost extinct because they are chased by herd owners.[2]

Many individuals remain in captivity in zoos, preserves and protected areas. Determining minimal nutritional requirements of large felines is difficult because their size and shape is not uniform and they also behave in a lonely way. A lack of full knowledge of these points, the

presence of risk factors and sometimes environmental conditions influence directly the presence of some diseases. Most notified diseases are those related to metabolic syndrome caused by non minerally balanced feed (bone diseases, teeth diseases and renal diseases) and obesity due to elevated glycemic load food intakes.[3]

Among infectious diseases viral pathogens that affect the respiratory system are the most common ones with the same clinical signals described in domestic felines. Stress disorders predispose felines to presentation of hidden infections that were non symptomatic or with nonspecific clinical signals, between them tuberculosis deserves special consideration for being zoonotic.[2]

Tuberculosis incidence in animal populations from zoos is low and the disease is surveilled with the intradermal test in primates and species that belong to the Order Artiodactyla.[4] To control the disease another tool as vaccination should be taken in count. Vaccination studies should be performed under conditions that mimic field situations so as to allow findings to be relevant to those situations.[5]

In other exotic species there is a risk because there are not available simple diagnostic tests to perform in field conditions. Researchers have diagnosed *M. bovis* using polymerase chain reaction (PCR) in fresh tissues from two leopards, although source of infection could not be established.[4]

One case of pulmonary tuberculosis caused by *M. bovis* subsp. *caprae* was described in a siberian tiger from Budapest Zoological and Botanic Garden. The specimen was obtained suctioning tracheal washing fluids which is a safe procedure for *in vivo* diagnosis. This method for obtaining clinical specimen in conjunction with routine differentiation and molecular methods allowed the rapid diagnosis of tuberculosis.[6]

In wildlife populations diseases caused by *Mycobacterium* sp. and related diseases can infect all vertebrate species from primates to fishes and bovine tuberculosis infecting wildlife Artiodactyla can be transmitted to other species including carnivores.[7]

Mycobacterial diseases diagnoses in wildlife species is not often carried out on clinical signals, meanwhile *postmortem* diagnoses is usually the first indicator of endemic and it is corroborated by laboratory tests. The prompt detection and effective management of infectious diseases in wildlife rely greatly on field diagnosis[4,7] because these infected animals are a likely source of infection that might spread microbial agents to other animal species including man.[8]

In the Kruger National Park, Mpumalanga Province, South Africa the prevalence of tuberculosis caused by *M. bovis* exceeds 70 % in African buffalo from the region and interspecies transmission (lion, cheetah, baboon, antelope) has also been confirmed.[9] In Wood Buffalo National Park from Canada the infection has been confirmed in wild wood bison and wild cervids populations, and also in 0.6 % of more than 600 wild birds.[8] In United Kingdom was described a resurgence of *M. bovis* infections in cattle and the difficulties in determining the sources of infection in those outbreaks. It was because mycobacterial transmission from animals without detectable tubercles and from wildlife still has not been well evaluated and the role of another important reservoir and source of infection as the *Homo sapiens* has not been well documented.[10]

In Czech Republic zoological gardens *M. bovis* from large felines has been isolated[11] and possible ways of transmission of mycobacterial infections through invertebrates and poikilothermic animals have been studied. In addition the impact of opportunistic mycobacteria on man and animals, and the course of the disease has been described.[12] *M. avium* subsp. *paratuberculosis* has been isolated from feral cats sampled around dairy farms on which paratuberculosis was present in cattle.[13] *M. simiae* has been cultured from a domestic cat that presented ocular manifestation and disseminated infection.[14]

Animal do not always act as the

source of infection of nontuberculous mycobacteria, these opportunistic bacilli are widely distributed in soil and water. These microbes have extraordinary starvation survival and tolerance of extremes temperatures.[15] A Greek study developed in tap water revealed the presence of *M. chelonae, M. gordonae*, *M. flavescens* and *M. terrae*. Environmental mycobacteria were harboured in water supplies and it has been demonstrated to be more resistant in general to chemical disinfectants than other bacteria.[16, 17] *M. scrofulaceum* and *M. avium* were isolated from tap water and have long been known as one agent responsible for cervical lymphadenitis in children, it is likely that children serve as sentinels for the presence of mycobacteria in water.[15]

A variety of techniques have been tested in hopes of improving nontuberculous mycobacteria recovery and identification. A definitive diagnosis of infection due to environmental mycobacteria has to be done on the basis of isolation and tipification of the microorganism and are also required pathological and hidstopathological findings to discount the possibility of contamination from the environment[18].

M. gordonae and microorganisms that belong to *M. avium* complex were the opportunistic pathogens mycobacterias most found throughout the United States. *M. gordonae* is ubiquitous and could be found in soil, water, nonpasteurized milk, human beings and animals mucous membranes, urine and gastric fluids, as its pathogenicity is very low its mortality rate is less than 0,1 % in immunocompromised patients.[19]

Nontuberculous mycobacterias cause opportunistic infections in persons that suffer from immune deficiency disorders and now are wide recognized in AIDS patients. Most recovered mycobacterias are *M. avium / intracellulare* and only a 9 % of cases are *M. tuberculosis*, wich is the most widespread pathogen among AIDS patients. That is why some opportunistic mycobacteria are considered among emerging infectious agents. Other less frecuent bacilli strains were *M. asiaticum, M. flavescens, M. fortuitum, M. gordonae, M. malmoense, M. scrofulaceum* and *M. xenopi* and some of them have been coinfective agents of *M. avium- intracellulare* and *M. simiae* infections. *M. simiae* strains differentiation is very difficult including the type strain because this bacilli behaves in a no reproducible way including highly standardized biochemical tests as photoreactivity or niacin test. The misidentification could include *M. simiae* as a specie member of MAIS.[20] Another aggravating factor is that pathogenicity of the strain in AIDS patients is not well understood.[16] It is important to highlight that transmission of *M. simiae* between animals in captivity appears possible.[18]

PCR-restriction analysis (PRA), combinate the amplification of a fragment of the *hsp*65 gene (heat shot protein) present in all mycobacteria, followed by restriction with *Bst*EII and *Hae*III of the PCR product, let the rapid identification of mycobacterial other than tuberculosis (MOTT).[21, 22, 23, 24]

This study was conducted to identify mycobacterial species isolated from the orpharynx of five American lions employing bacteriological and molecular biology techniques. The animals under study did not show specific clinical signals and were from a wildlife preserve that did have feline tuberculosis background.

Material and Method

The five American lions under study were from a wildlife preserve in the southeast of Buenos Aires Province, Argentina wherein has occurred a case of tuberculosis in an adult American lion. That animal showed tuberculosis clinical signals and pathological lesions in which ones acid-alcohol-fast bacilli could be seen when Ziehl-Neelsen (ZN) staining was performed. The samples were swabs from the oropharynx of the five American lions that shared smallholding with the diseased feline. Before taking the material to be tested the animals were anesthetized by the veterinary surgeon that assessed the natural preserve and all guidelines and rules for animal welfare were

observed during the experience.

The specimens were stained with ZN and inoculated in Löwenstein Jensen and Stonebrink culture media. Mycobacterial differentiation was done on the basis of: rate of growth, growth at 25ºC, 37ºC and 45ºC, carotenogenesis constitutive and photoinducible, nitrate reduction test, semiquantitative catalase test, stability of catalase to 68°C, hydrolisis of Tween, urea lytic activity, intake of iron, growth on MacConkey agar and on medias containing 5 % of NaCl, hydroxylamine and 0,2 % of picrate.[25]

To detect members of the *M. tuberculosis* complex polymerase chain reaction was applied to amplify insertion sequences IS*6110* and IS*1081*[26] and was also amplified *hsp65* wich is present in all mycobacterias. Amplification product from *hsp65* was digested with restriction enzymes *BstE*II and *Hae*III accordingly to PCR restriction analyses (PRA) and the fragments obtained were separated by electrophoresis on a 3 % agarose in 1x TBE buffer.[24] The patterns were compared with those included in the database PRASITE (http://app.chuv.ch/prasite).

Results

Smears from all specimens were stained with ZN acid fast procedure and microscopically examinated, four of them presented acid fast bacilli. Specimens were decontaminated and inoculated in Stonebrink and Löwenstein-Jensen media, they were incubated before and five strains slowly growing were obtained. The strains were able to develop at 25 and 37 ºC but could not do it at 45ºC. Four of the five strains isolated produced pigmentation in the dark (scotochromogenous) and one of them did not present carotenogenesis (non chromogenous). Mycobacterial species were identified as *M. gordonae* (two strains), *M. simiae* (one strain), *M. scrofulaceum* (one strain) and *M. avium/intracellulare* (one strain) performing those tests described previously.

The strains did not amplify IS*6110* y IS*1081*, but using PRA technique were identified *M. gordonae* patterns in three strains using different algorithms and tables. Differentiation between *M avium* III or *M. simiae* III patterns in two strains could not be done because the size of the restriction fragments gave the same patters in both of them. Results are presented in table 1 and figure 1.

Discussion and Conclusions

Pathological investigation in wild animal is usually supported in gross *post mortem* examination and histopathology, haematology, cytology and serology but not in the confirmation of a case by the isolation and identification of the agent. Generally the diagnosis of *Mycobacterium* related diseases in wildlife is done on the basis of finding tuberculous lesions during *post mortem* examination and looking at their histopathologycal changes and the presence of acid fast bacilli with Ziehl Neelsen

Table 1 - Identification of mycobacterial species using PRA and bacteriological tests - tandil, Buenos Aires, Argentina - 2007

Samples	Restriction patterns (bp) *BstEII*		*HaeIII*			PRA results	Bacteriology results
Sandokan	240	210	145	130		*M.avium* III / *M. simiae* III	*M. gordonae*
Linda 1	325	120	130	115	60	*M. gordonae* IV	*M. scrofulaceum*
Linda 2	325	120	130	115	60	*M. gordonae* IV	*M. gordonae*
Julio	240	210	130	115		*M. gordonae* V	*M. avium / intracellulare*
Luisa	240	210	145	130		*M.avium* III / *M. simiae* III	*M. simiae*

Sandokan, Linda1, Linda 2, Julio and Luisa were the names of the strains isolated during laboratory schedule

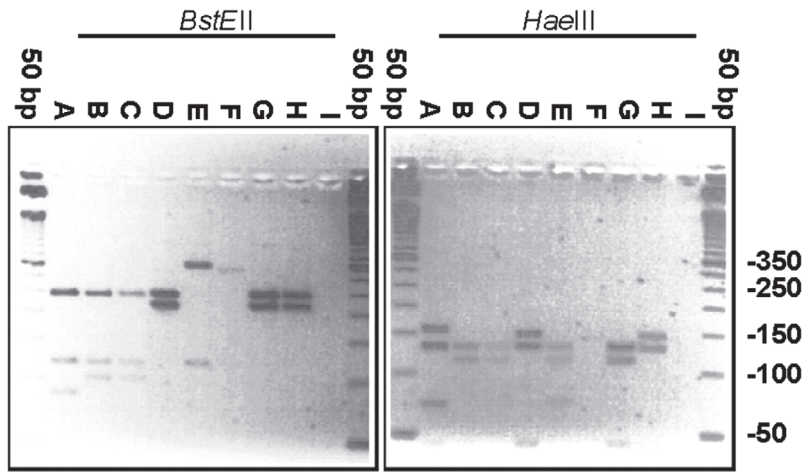

Figure 1 - Electrophoresis gel of DNA restriction fragments obtained with *BstE*II and *Hae*III enzymes - Tandil, Buenos Aires, Argentina - 2007. Lane A) *M. bovis* AN5; Lanes B and C) *M. gordonae* III; Lane D) Sandokán; lane E) Linda 1; Lane F) Linda 2; lane G) Julio; Lane H) Luisa and Lane I) PCR negative control

staining.[3] In the present surveillance study we have isolated and classified until species level the agents by biochemical and molecular methods. However we were not able to perform the pathological investigation because the animals under study were kept alive in the zoological garden.

Arriving to species level in the taxonomic classification also allowed the differentiation of *M. avium* and *M. simiae* from *M. bovis*, all of these microbial species are non chromogenous slow growing acid fast bacilli.[25] The information mentioned above is also decisive for veterinary surgeons if they intend to implement antimicrobial therapy when disease is confirmed because some species as *M. avium* are notoriously resistant to anti-mycobacterial drugs.[27]

All the strains did not amplify IS*6110* and IS*1081* and PRA test allowed the identification of *M. gordonae* patterns in three strains and *M. avium* III or *M. simiae* III patterns in two strains, meanwhile using bacteriological tests for species identification two strains were *M. gordonae* and the other strains were *M. scrofulaceum, M. avium / intracellulare* and *M. simiae*. Bacterial identification by biochemical tests and molecular biology techniques matched in two strains but did not on the other three, that is why it is very important to employ them as multiple tests. As nontuberculous mycobacteria are very concerning to public and animal health research and understanding on this subject is needed.

All strains identified are included in the group of nontuberculous or environmental mycobacteria which has a significative public health meaning among immunocompromised patients and economic meaning to livestock industry.[15]

Mycobacterial species isolated during this study have been isolated from generalized syndrome in immunocompromised AIDS patients. Reports of the occurrence of lentiviruses closely related to feline immunodeficiency virus in a variety of nondomestic felids worldwide have been done and also the presence of feline leukemia virus infection in single case reports.[28] The immunosupresive effect caused by these viral agents in wild felines could probably be a predisposal factor as HIV is to human populations.

Another cause of economic loss to animal populations from livestock industry due to mycobacterial diseases is the transmission causing non-specific positive reactions to tuberculin skin testing in cattle.[29,30] Indirect losses may be less obvious but far-reaching, these include loss of valuable endangered species as are wild felines.[29, 30] To preserve ecological heritage it is important to avoid *Felis concolor* indiscriminate chase as

happens in the pampa land from Argentina, where herd owners pursue American lion because are predatories.

In zoological gardens and natural preserves mycobacterial diseases represent a serious threaten to biodiversity and poses a regulatory dilemma in translocation exercises.

The role of feral cats from America land as *Felis concolor* in the epidemiology of diseases caused by nontuberculous mycobacteria is unclear and remains to be further investigated.

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Isolamento de micobactérias em *Felis concolor* em cativeiro

Resumo

Este trabalho foi realizado em uma reserva natural da Argentina com antecedentes de tuberculose em uma suçuarana adulta. O objetivo foi identificar por meio de técnicas bacteriológicas e de biologia molecular as espécies isoladas da orofaringe de cinco suçuaranas que apresentavam sinais clínicos inespecíficos. As amostras foram colhidas das suçuaranas após sedação. Posteriormente foram processadas para obtenção do isolamento e identificação por meio de provas bioquímicas do gênero *Mycobacterium* pela técnica de PCR. Investigou-se a presença das seqüências de inserção IS*6110* e IS*1081* e hsp*65*. Obtiveram-se resultados positivos à coloração de Ziehl-Neelsen de quatro amostras, isolando cinco cepas de crescimento lento. As cepas foram classificadas como *M. gordonae* em dois casos e *M. simiae*, *M scrofulaceum* e *M. avium/intracellulare* em um. Por PRA, identificou-se o padrão de *M. gordonae* em três cepas e *M. avium III* ou *M. simiae* em dois.

Palavras-chave:
Felis concolor.
Jardim zoológico.
Micobactérias não tuberculosas.

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