REPORT OF MYCOBACTERIA ISOLATED FROM DOMESTIC AND WILDLIFE SPECIES DURING 2004-2008

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ABSTRACT: *Detection and identification of bovine tuberculosis and its differentiation from micobacteriosis is fundamental during diagnoses. That is why mycobacteria laboratories improvement becomes essential in public health and veterinary medicine services. The objective of the present research is to differentiate Mycobacterium bovis and nontuberculous mycobacterias in isolates cultured from domestic and wildlife species from seven Argentinean provinces during 2004-2008. Differentiation was based on biochemical tests, phenotypic characteristics and M. bovis spolygotyping. Biochemical and phenotypic identification resulted in 20 M. bovis strains, 18 of them were confirmed by spolygotyping, and 34 nontuberculous mycobacteria strains. Thirteen species were characterized and all of them were grouped considering biological risk and pathogenic potential reported in humans and/or animals. Here we have reached advances in tuberculosis and micobacteriosis diagnoses in veterinary medicine. In this area diagnoses are often based on micro and macroscopic observation of the tubercles and skin test results. These advances are not minor as zoonotic tuberculosis is still a public health problem in Latin America.*

Keywords: *Mycobacterium*, report, animals, wildlife, domestic

MICOBACTERIAS AISLADAS DE ESPECIES DOMÉSTICAS Y SILVESTRES DURANTE 2004-2008

RESUMEN: *La identificación de la tuberculosis bovina y su diferenciación de las micobacteriosis es fundamental durante el diagnóstico. Es por eso que los laboratorios especializados en micobacterias son de suma importancia en los servicios de salud pública y salud animal. El objetivo de la presente investigación es diferenciar Mycobacterium bovis de micobacterias no tuberculosas en cepas cultivadas a partir de especies domésticas y silvestres de siete provincias de Argentina durante 2004-2008. La diferenciación se basó sobre las pruebas bioquímicas, las características fenotípicas y el "spolygotyping" de M. bovis. Con la identificación bioquímica y fenotípica se detectaron 20 cepas de M. bovis, 18 de las cuales fueron confirmadas mediante "spolygotyping", y 34 cepas de micobacterias no tuberculosas. Trece especies fueron caracterizadas y todas ellas fueron agrupadas considerando el riesgo biológico y el potencial patógeno notificado en seres humanos y/o animales. En este trabajo se han logrado avances en el diagnóstico de tuberculosis y micobacteriosis en medicina veterinaria. En este área el diagnóstico habitualmente se basa sobre la observación micro y macroscópica de los tubérculos y los resultados de la intradermorreacción. Estos avances son importantes porque la tuberculosis zoonótica aún es un problema de salud pública en América Latina.*

Palabras clave: *Mycobacterium*, notificación, animales, silvestres, domésticos

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INTRODUCTION

Mycobacteria are causative microorganisms of tuberculosis in human beings and animals, they also cause the disease called micobacteriosis (1, 2). The genus *Mycobacterium* can be divided into three groups based on clinical implications, the first one includes strict pathogens as the members of the *M. tuberculosis* complex. The second group consists of mycobacteria potentially pathogens and the third is composed by non-pathogenic or exceptionally pathogenic species also known as saprophytic mycobacteria. Second and third group species are often literary referred to as nontuberculous mycobacteria (NTM), paratuberculous mycobacteria, anonymous mycobacteria, mycobacteria other than tuberculosis or atypical mycobacteria (1, 2, 3).

Identification of *Mycobacterium* strains to species level has been traditionally based on the results of biochemical tests and phenotypic characteristics like growth rate and pigmentation. Although most of these tests are simple to perform and do not require sophisticated equipment, their results are often delayed because mycobacteria present low replication rate. These can be a serious trouble in the clinical field of public health (2), but it is not the same in animal health wherein samples often come from dead animals (4). Nevertheless traditional methods constitute the main procedure for mycobacteria identification, especially in countries with low economic resources (2, 4).

During last years methods that analyse microbial genetics have been improved, they often detect highly conserved regions within the genome that harbour hypervariable sequences in which species-specific events are present (2). Mycolic acids profiles obtained with chromatographic techniques are also used as genera chemotaxonomic traits (1). Moreover mycobacteriophages are employed as diagnostic markers to improve and expedite the recognition of pathogenic mycobacteria as well as their drug resistance (5).

Even though some mycobacteria laboratories in veterinary medicine have improved *M. bovis* and NTM detection and identification, bovine tuberculosis is still a health problem in non-industrialized regions and its differentiation from micobacteriosis is fundamental during differential diagnosis. The objective of the present research is to identify *M. bovis* and NTM in isolates cultured from domestic and wildlife species from seven Argentinean provinces during 2004-2008.

MATERIALS AND METHODS

SAMPLES AND BACTERIAL ISOLATION

During this study acid fast bacilli (AFB) were isolated in seven animal health research centres. Samples were obtained from dairy and beef cattle farms that presented positive reactors to the tuberculin skin test and consisted of nasal swabs, milk and tissue from necropsy material. Samples from wildlife species were tissue from necropsy material taken following animal welfare protocols. Sampled animals shared the habitat with the animal population of selected farms.

To obtain mycobacteria strains samples were processed and decontaminated using Petroff protocol. Culture was performed at 37 °C in Löwenstein-Jensen and Stonebrink solid media and speed of growth was registered weekly (6, 7, 8).

Bacterial primary isolations obtained in each research centre were transported to identification laboratory properly stored following biosecurity recommendations (8, 9).

PHENOTYPIC IDENTIFICATION

To confirm the presence of AFB in purity smears were stained with Ziehl-Neelsen method and were observed with optic microscope oil immersion objective (10). Then colonies aspect and pigment production were verified, when colonies were not pigmented it was registered if eugonic growth was present in Stonebrink or Löwenstein-Jensen media. This practice focused *M. bovis* search because it presents eugonic growth only in Stonebrink media (6, 7, 8, 9).

Pigment production and optimal temperature of growth tests were done in all strains under study. The information obtained in those tests was used to include each strain in a Runyon group.

BIOCHEMICAL IDENTIFICATION

To verify the presence of *M. bovis* differentiation protocol proposed by de Kantor and Bernardelli in 1987 (11) was employed and NTM were characterized with the biochemical tests sequences suggested by de Kantor (6, 7), Thorel *et al.* (12) and Lévy-Frébault & Portaels (13). Since 2005 flow charts and interactive tables developed by Leao *et al.* had been implemented (2).

SPOLIGOTYPING

To genotype 18 *M. bovis* isolates the cultures were subjected to spoligotyping as Kamerbeek *et al.* (14) has described. Briefly a loopfull of bacteria was suspended in distilled water and boiled for 30 minutes. After centrifugation supernatant was used for PCR to perform spoligotyping (Ocimum Biosolutions B. V.). Clusters of isolates were defined as two or more *M. bovis* strains with identical spoligotypes. Each spoligotype was allocated with a number according to data base of Biotechnology Institute, INTA-Castelar.

RESULTS

IDENTIFICATION OF MYCOBACTERIA

A total of 68 bacterial strains were isolated in Santa Fe (*n:* 21), Buenos Aires (*n:* 17), Córdoba (*n:* 17), Corrientes (*n:* 8), Entre Ríos (*n:* 3), Catamarca (*n:* 1) and Tucumán (*n:* 1) provinces, from them 56 belonged to bovines, nine to wildlife mammalians, two to birds and one to a goat. During microscopic examination AFB were evidenced in 54 smears and considered as bacterias from *Mycobacterium* genus. The 14 negative AFB smears were not considered as mycobacteria. Biochemical and phenotypic identification resulted in 20 *M. bovis* strains and 34 NTM strains. Thirteen species were characterized and all of them were grouped considering biological risk and pathogenic potential reported in humans and/or animals (Table 1).

M. bovis ISOLATES

M. bovis strains were isolated from 16 bovine necropsy material that presented granulomatous lesions, in one case it was isolated from bovine nasal swabs and in another from bovine milk. From the information recorded in protocols filled during sampling it was corroborated that in 16 cases strains were isolated from positive reactors to skin test, in one case the test gave negative result and in another it was not performed. One strain of *M. bovis* was characterised from a granulomatous lesion diagnosed in a deer. The last *M. bovis* strain was isolated from a wildlife specie that could not be determined because the sender did not identify the sample in the sampling protocol.

Eighteen *M. bovis* strains were genotyped by spoligotyping and 6 different spoligotypes were identified. Four of them were grouped in clusters as follows: Spo 47 (*n:* 7), Spo 21 (*n:* 4), Spo34 (*n:* 3) and Spo 75 (*n:* 2). Spoligotypes Spo 17 (*n:* 1) and Spo 96 (*n:* 1) were presented solely (Figure 1).

NTM ISOLATES

NTM were isolated from 28 bovines, five wildlife species and one goat. Mycobacteria species were *M. smegmatis* (*n:* 9); *M. shimoidei* (*n:* 4); *M. phlei y M. avium/intracellulare* (*n:* 3); *M. chelonae*, *M. chitae*, *M. flavescens*, *M. nonchromogenicum*, *M. szulgai* y *M. thermoresistibile* (*n:* 2) y *M. malmoense, M. gastri* y *M. terrae* (*n:* 1).

DISCUSSION

Although in Argentina tuberculosis prevalence has declined (15, 16) *M. bovis* is still cultured from bovine tubercles found in positive reactors and during this research it has also been detected in a tubercule from a cervid. White-tailed deer can act as a maintenance host for *M. bovis* being a source of infection to bovines (17) and human beings (18).

M. bovis biochemical characterization was corroborated by spoligotyping and the biggest cluster corresponded to spoligotype Spo 47 which was found in a particular herd of Córdoba province and up to date it was not found in any other province. Spoligotypes Spo 34 and Spo 21 are the most frequent in Argentina groupping 47 % and 20 % of the isolates respectively (19).

As information about bovine tuberculosis status is scarce in most developing nations *M. bovis* results found in this research are fundamental in public and animal health. Moreover isolation and identification until specie level is substantial to confirm infection when abattoir surveillance methods are applied (20).

During the present report *M. smegmatis* was characterised from lesions resembling tubercles, also were *M. chelonae* and *M. phlei*. Bercovier & Vincent (21) reported *M. smegmatis* y *M. chelonae* from granulomatous lesions in bovines and Hines *et al.* (22) focused that *M. phlei* was the cause of infection in domestic carnivores.

M. smegmatis was also isolated from bovine milk, Schultze *et al.* (23) have diagnosed this mycobacteria as a cause of bovine mastitis. In mastitis to achieve specie level characterization is decisive for veterinary surgeons if they intend to implement specific antimicrobial therapy when disease is confirmed.

From bovine necropsy material it was also typified *M terrae*, a slow grower mycobacteria reported to cause non specific tuberculin reactions to bovines (24, 25). Non specific reactions increase when bovine tuberculosis is progressively eradicated from a country (20).

Spoligotype Number	Spoligotype	No of isolates
47		7
21		4
34		3
75		2
17		1
96		1
Total		**18**

Figure 1. Representation of 6 spoligotypes identified among 18 *M. bovis* isolates studied.

Table 1. Phenotypic and fundamental characteristics of Mycobacterium species under study

MYCOBACTERIUM SPECIE	RISK LEVEL	HUMAN BEINGS REPORTED INFECTION	ANIMALS REPORTED INFECTION	SPEED OF GROWTH	PIGMENT PRODUCTION	RUNYON GROUP
M. bovis	III	Yes	Yes	Slow	Not pigmented	III
M. avium/ intracellulare	II	Yes	Yes	Slow	Not pigmented	III
M. flavescens	I	No	No	Slow	Schotochromogen	II
M. gastri	II	Yes	No	Slow	Not pigmented	III
M. malmoense	II	Yes	No	Slow	Not pigmented	III
M. nonchromogenicum	I	No	No	Slow	Not pigmented	III
M. shimoidei	II	Yes	No	Slow	Not pigmented	III
M. szulgai	II	Yes	No	Slow	Schotochromogen/ Photochromogen*	I / II
M. terrae	I	No	No	Slow	Not pigmented	III
M. chelonae	II	Yes	Yes	Fast	Not pigmented	IV
M. chitae	I	No	No	Fast	Not pigmented	IV
M. phlei	I	No	Yes	Fast	Schotochromogen	IV
M. smegmatis	I	Yes	Yes	Fast	Not pigmented	IV
M. thermoresisitibile	I	Yes	Yes	Fast	Schotochromogen	IV

*Schotochromogen at 37°C and photochromogen at 25 °C

Although NTM were isolated from tubercle like lesions we are not able to induce that those animals were mycobacteria infected or diseased because pseudoinfection during collection of samples may occur. Moreover in animal health it is a real possibility because bovine necropsies are performed by veterinary surgeons in the field so samples in aseptic conditions are not easy to obtain. Pseudocontamination can occur during the course of analysis in the laboratory, and is more frequent if the strains are processed in batches (2). We made laboratory work in batches, but including in the biosecurity cabinet open plates with micobacteria specific media to detect cross contamination. Those plates were incubated and followed with each batch of strains.

In veterinary medicine it is difficult to deepen in pathological studies because samples often come from *post mortem* lesions so it is not easy to perform repeated isolation. This is one necessary requirement to corroborate a NTM as the cause of disease (26) that is why research in experimental models reproducing mycobacteriosis are needed as is the case of the *M. fortuitum* infection model (21).

Diagnoses of tuberculosis and micobacteriosis in animal populations are often based on micro and macroscopic observation of the tubercles and tuberculosis skin test results. In the present research advances have been reached because primary isolations and species level differentiation were done. These are not minor goals because zoonotic tuberculosis is still a health problem in some Latin American countries (16) wherein the majority of the economies are emerging or developing ones with scarce access to the technologies currently used by mycobacteriologists in high income countries.

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