University of Veterinary Medicine
Doctoral School

Identification and typing of non-tuberculous Mycobacteria

Brief Summary of Doctoral Thesis

dr. Zsuzsanna Rónai

2016

Supervisor and consultants:

Dr. Ádám Dán
National Food Chain Safety Office,
Veterinary Diagnostic Directorate,
Molecular Biology Department
Supervisor

Dr. Sándor Tuboly
University of Veterinary Medicine
Department of Microbiology and Infectious Diseases
Consultant

Dr. Miklós Gyuranecz
Hungarian Academy of Sciences,
Centre for Agricultural Research,
Institute for Veterinary Medical Research
Consultant

Introduction, Aims of the Study

One of the most important goals of the Hungarian livestock breeding in the past few years was to achieve the official declaration of the tuberculosis-free status of our cattle farms. Although the cattle herds in Hungary have been free of bovine tuberculosis since 1981, the European Union has not recognized it due to the high number or reactive animals. Herds, in which the presence of tuberculosis could not be verified, but in tuberculin skin testing numerous positive or inconclusive reactions occurred, were certified as 'paraallergic'. Due to the high degree of antigenic relatedness non-tuberculous Mycobacteria (NTM) infected animals can react in the tuberculin skin testing, thus hampering the *in vivo* diagnosis of bovine tuberculosis.

The primary aim of our study was the identification and typing of NTM in Hungary. This group of mycobacteria is very diverse. The culture and growth conditions of the different species are varied, which poses a challange to the laboratory veterinarians.

In order to achieve this goal molecular biological identification methods had to be introduced and developed in addition (and partly as replacement) to the previous classical diagnostic procedures.

Following the accurate identification of the *Mycobacterium* strains we intended to map the most common strains and identify their host species spectrum and pathogenicity.

We also wanted to test the genetic diversity of the *M. avium* strains, and explore the epidemiological relationships between the different genotypes.

From the strains we aimed to set up a strain collection, which could later be used as a basis for future studies.

Materials and Methods

The diversity of the samples and the *Mycobacterium* strains did not allow us to follow a strict sample processing protocol, so we developed operational protocols for the different sample types and *Mycobacterium* strians.

In case, a strain could not effectively be grown on the surface of solid media, or selective isolation from mixed culture was necessary, by combining various additives and raw materials we developed new culture media.

The inoculated solid media were checked by daylight with the naked eye, while from the liquid media Ziehl−Neelsen (ZN) stained smears were prepared. The MGIT tubes were only stained if the Wood-lamp test gave positive results.

At the examination of the smears we observed not only the occurrence of ZN-positive bacteria, but noticed also their size, form, relative location (cord formation, clumps), and the presence of contaminant bacterial flora.

In case of ZN positive cultures we recorded the incubation period until the strain appeared on the surface of solid media, and observed its primary characteristics, like colony morphology. From clean cultures subcultures were prepared for further studies. Liquid cultures or contaminated cultures were decontaminated before subculturing to remove the contaminant flora.

Type of media inoculated at subculturing and the conditions of incubation were choosen on the basis of the characteristics of the primary culture. A medium, on which the strain was originaly isolated, was in each case inoculated at subculturing. Subcultured media were observed every second day. In case of bacterial growth ZN staining was performed to check the purity of the culture. From subculture strains we receroded the growth rate (rapid growers: within 7 days, slowly growing strains: over 7 days), temperature tolerance, pigment production, colony morphology and culture medium preference.

The cultured *Mycobacterium* strains were further investigated using molecular biological methods. The prerequisite for successful molecular biological studies is that from the samples bacterial DNA in appropriate quantity and quality should be extracted. Due to the special cell-wall of *Mycobacteria*, the simple freeze/thaw DNA extraction method, which works perfectly with other bacteria, does not provide sufficient DNA amount. In fact, the different sample types (sputum, tissues, faeces) and bacterium strains also require various extraction methods.

For our cultures we used sonication and boiling or manual DNA extraction according to an in-house protocol.

In our study we have attempted to detect and identify *Mycobacteria* directly from the samples, which due to the low bacterial load and the presence of potential inhibitory substances required specific DNA extraction methods. For organ and tissue samples we used Roche High Pure PCR Template Preparation v.16.0 kit and QIAamp® DNA Mini kit, while for faecal samples we also used ExtractMaster™ Fecal DNA Extraction kit, according to the manufacturer's instructions.

In the different PCRs beside extraction negative and PCR negative controls *M. bovis* CIP102426^T, *M. avium* subsp. *avium* NCTC13034^T, *M. avium* subsp. *silvaticum* ATCC49884^T and *M. avium* subsp. *paratuberculosis* ATCC19851 strains were used as PCR positive controls.

For the discrimination of *M. avium* subsp. *avium* (MAA) and *M. avium* subsp. *silvaticum* (MAS) strains we developed a molecular biological identification method, which uses subspecies specific mismatch (MM) primers and high-resolution melt (HRM) analysis in a duplex assay.

PCR products for sequencing were extracted from agarose gel by QIAquick Gel Extraction Kit. In the sequencing reactions the same primers were used as in the PCRs, except the 16S rDNA, where beside the 27F and 1492R amplification primers an extra sequencing primer (536F) was applied.

The sequencing reactions were performed by BigDye® Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's instructions. The sequence runs were accomplished by Biomi Kft. on an ABI Prism 3400 DNA Sequencer apparatus.

Sequence reads from two directions were assembled by SeqMan – Lasergene 12 (DNASTAR) program. Sequences for pairwise comparison were aligned in BioEdit 7.2.5, while philogenetic analysis was performed in MEGA 6.06 szoftver with Neighbor-Joining (NJ) method.

The unique sequences detected during our study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank) online database.

Genotyping of the *M. avium* subspecies was performed by LSP^{A}17 sequence polimorphism and MIRU−VNTR analyses.

MIRU−VNTR typing was performed by amplification of four MIRU, three VNTR and one MATR loci. The number of tandem repeats (TR) was determined from the size of the amplicons. In cases of small allelic differences PCR products were sequenced.

The allelic diversity (h) of the different loci was calculated by the equation $h = n(1 - \Sigma x_i^2)/(n-1)$, where n is the number of bacterial strains and x_i is the frequency of the i^{th} allele at the locus. The Index of Discrimination (DI) was determined according to the formula described by Hunter and Gaston. Epidemiologically related strains were excluded both from allelic diversity calculation and DI determination.

In order to determine the genetic relatedness of the different genotypes Neighbour-Joining (NJ) analysis was performed in MEGA6.

Results

Between 2006 and 2015, almost 3000 *Mycobacterium* strains were isolated at the Bacteriological Laboratory of the National Food Chain Safety Office – Veterinary Diagnostic Directorate. After determining their main group-type, further studies were undertaken. From the nearly 2400 NTM almost 1000 proved to be *M. avium*.

Among the *M. avium* strains 144 MAA, 65 MAS, 94 MAH and 659 MAP were identified.

Mycobacterium avium subsp. *avium* (MAA)

MAA strains were isolated besides domestic, wild and zoo birds from domestic and wild mammalian species, with the exception of Heves and Tolna Counties from the whole geographic region of Hungary. MAA infection in red foxes was reported first.

In birds we observed the post-mortem picture of avian tuberculosis without exception, against the red deer and red fox samples, which contained no visible lesions.

The cattle, wild boar and swine samples showed macroscopic pathological changes in 14.3, 54.5 and 95.7% respectively. Swine samples originated from 27 farms of 8 different Counties, while cattle samples came from 12 farms from 9 Counties. Cattle hosts reacted in tuberculin skin test with one exception. Five strains were grown from dairy cows, while 9 from beef cattle. Age of host animals was between 11 months and 14 years, which were heifers with one exception.

Mycobacterium avium subsp. *silvaticum* (MAS)

MAS strains were isolated for the first time from wild boars, red fox, red deer, badger and cattle.

Macroscopic lesions were found in 35% (23/65) of the samples, 60% (14/23) of which showed histological changes characteristic of tuberculosis. Cattle hosts were Limousine and Hungarian Piebald breeds, and gave positive reactions in tuberculin skin testing.

The developed real-time PCR assay correctly identified all 65 MAS strains, which eliminated the long-existing problem of the ambiguous phenotypic identification.

The MM primer targeted a C/G SNP in the *asp*B gene. This PCR gave a 369-bp-long MAS specific amplification product with a Tm of 94.9°C, while no products appeared with MAA, MAH, MAP, *M. bovis and M. intracellulare* strains. The HRM primer pair was designed to contain a TT/CG difference in a putative membrane protein gene. The PCR products of MAA (T_m: 91.63) and MAS (T_m: 91.17) strains had unique melting-curve profiles in the normalized graph, with a temperature shift of 0.7°C. The independent-sample t test confirmed that the results of the HRM analysis were statistically significant ($P<0.0001$). The input DNA template range extended between 100ng and 15pg.

The PCR products obtained by using the MM and HRM primers were sequenced to confirm the presence of the sequence variations. The sequences were deposited in GenBank under accession numbers KP792232−KP792236.

Mycobacterium avium subsp. *hominissuis* (MAH)

Besides wild and domestic mammals MAH strains were isolated in one case from a dog and in two cases from zoo birds, with the exception of Fejér County from the whole geographic region of Hungary.

The infected swine hosts came from 8 different farms in 7 Counties, and displayed characteristic lesions in their mesenteric lymph nodes without exception.

Cattle hosts originated from 38 farms in 16 Counties, and reacted in tuberculin skin testing at 76% (35/46). Two thirds of the strains were isolated from dairy (Holstein-Friesian, Jersey) cattle, while one third from beef (Aberdeen Angus, Charolais, Hungarian Grey Cattle) cattle. Age of host animals was between 3 months and 13 years. Only seven strains were isolated from bulls, 39 from heifers.

Among the red deer and wild boar samples macroscopic lesions were found at 12.5 and 28.6%. The peacock and turaco showed the characteristic post-mortem picture of avian tuberculosis. The host of our dog strain was a 2 years old male miniature Schnauzer, in which systemic mycobacterial infection was diagnosed with enlarged lymph nodes throughout the body.

Mycobacterium avium subsp. *paratuberculosis* (MAP)

MAP strains were isolated in large numbers from the whole geographic region of Hungary. Besides domestic and wild ruminants the pathogen was also isolated from wild boars, red fox and swine. This is the first time in Hungary that MAP strains were typed. Presence of type I (sheep type) strains in sheep, goats and cattle were detected. We isolated type II (cattle type) strain from swine for the first time.

Only 9.7% of the strains (64/659: 3 sheep, 4 goats, 4 muflon, 53 cattle sample) came from samples sent in the laboratory with the explicit purpose of MAP cultivation. In the remaining cases paratuberculosis was diagnosed as subsidiary finding in the course of bovine tuberculosis bacterial culture tests.

From the 542 strains, which were found in cattle as subsidiary findings, 76 (14.02%) came from animals, which did not reacted in tuberculin skin testing. The hosts of the remaining 466 strains (85.98%) gave positive or inconclusive reactions in tuberculin skin tests. From beef cattle (Aberdeen Angus, Aubrac, Charolais, Limousin, Hungarian Gray Cattle) 158, from dairy cattle (Brown Swiss, Holstein-Friesian, Jersey) 416 strains originated, which came from 184 different farms. The youngest host was 2 months old, while the oldest 16 years old.

7.6% (44/577) of the strains with known origin came from bulls and 92% from heifers. MAP stains were isolated at multiple cases from hosts with family relations, e.g.: mother and calf, brother/sister calves; and from animals imported from foreign coutries like Germany, Denmark, Slovakia and the Netherlands.

The buffalo, red deer, red fox and swine hosts came from Somogy County. The infected mouflons were imported from the Czech Republic and were sampled in quarantine. The sheep came from Hajdú-Bihar and Jász-Nagykun-Szolnok Counies, while the goats from Hajdú-Bihar, Pest, Somogy and Veszprém Counties. The majority of the wild boars (22/31) were hunted in Somogy, but some animals came from Bács-Kiskun, Baranya, Győr-Moson-Sopron, Hajdú-Bihar, Heves, Komárom-Esztergom, Tolna, Vas or Veszprém Counties.

From 41 faecal, 4 goat gut and 3 sheep gut samples, sent into the laboratory for targeted detection of paratuberculosis, we tried to detect MAP directly with specific PCR assays after different DNA extractions. With bacterial culture, which is considered the 'gold standard', we could isolate 19 strains. Samples, which were culture negative, were also tested negative in the direct PCRs. From the positive samples 15 were found positive with PCR. The best performance was achieved with Roche High Pure PCR Template Preparation v.16.0 kit and ExtractMaster™ Fecal DNA Extraction kit and F57 MAP specific PCR.

Detection and Identification of non *M. avium* NTM

In addition to the nearly 1000 *M. avium* strains 1419 other, non *M. avium* NTM were identified between 2006 and 2015. More than one third of these strains were cultured from milk samples. For further investigations 230 strains were selected, of which 38 were of milk origin.

Red deer strains were isolated from Somogy County except two (Tolna). We had strains from wild boars from whole Hungary except the Counties on the Great Plain (Bács-Kiskun, Békés, Csongrád, Jász-Nagykun-Szolnok). Infected swine came from a pig farm in Baranya County. The 103 cattle strains came from 65 different herds, from bulls (12%) and cows (88%), beef (33%) and dairy (67%) cattle. However, 92.3% of the hosts reacted in tuberculin skin testing; pathological changes were only found in 7.7% of the samples. The strains from milk samples originated from 17 different herds in 8 Counites.

Within the tested strains *M. nonchromogenicum* was the most frequent (25.2%), followed by a 16.5% presence of *M. smegmatis*. Strains, like *M. fortuitum*, *M. intermedium* and *M. kansasii* were isolated repeatedly, while others like *M. chitae*, *M. chelonae*, *M. parafortuitum* or *M. nebraskense* only in single cases.

While strains like *M. abscessus*, *M. palustre*, *M. peregrinum* or *M. thermoresistible* were identified in different host species, we only found *M. smegmatis*, *M. neoaurum*, *M. shimoidei*, *M. europaeum*, *M. nebraskense*, *M. malmoense* and *M. phlei* in cattle, *M. arosiense*, *M. saskatchewanense* and *M. gordonae* in wild boars and *M. parafortuitum* in red deer.

97% (100/103) of the cattle strains of non-milk origin could be identified. The remaining 3 strains (*M. sp.* #4) had unique and identical *tuf*, *rpo*B and 16S rDNA sequences. Such strains were also isolated from two red deer samples.

Among the strains isolated from wild boars 45 known species (88.2%) were identified. The remaining 6 strains represented new types (*M. sp.* #1, #2, #3, #6, #7, #8). From the 16 red deer strains 9 could be identified. The unidentified strains represented 4 different new types (*M. sp.* #3,#4, #5, #7). Sequences of the strains, which could not be identified on the basis of their *tuf*, *rpo*B or 16S rRNA genes were deposited in the GenBank with the accession numbers KP840553−KP840591. Strains from swine, roe deer, fallow deer, red fox, sheep, dog, camel, chameleon, crocodile and a human could be identified without exception.

From the commercially available kits, INNO-LiPA MYCOBACTERIA v2 could only identify 10% of our strains in a preliminary study conducted on 50 strains; thus this method was excluded from further investigations. The GenoType Mycobacteria CM/AS kit misidentified the *M. bourgelatii* and some *M. intermedium* strains as *M. gordonae*, the majority of the *M. nonchromogenicum* strains as *M. celatum*, two *M. peregrinum* strains as *M. fortuitum*1, 12 *M. smegmatis* strains as *M. fortuitum*2, and three new strain types as *M. szulgai, M. scrofulaceum* and *M. intracellulare*. With this kit the rate of correct identifications reached only 22.07% (34/154).

The majority of the tested strains was clearly identifiable on the basis of their *rpo*B or *tuf* gene sequences, while 16S rDNA did not prove to have appropriate resolution.

Our *M. ulcerans* ecovar Liflandii strain showed high similarity to *M. ulcerans* and *M. marinum,* and could not be identified with 16S rDNA, *tuf* and *rpo*B sequences either. Based on literature data we tested IS*2404* and the presence of *esx*A and *esx*B genes and the partial sequence of *hsp*65.

From 34 representative *Mycobacterium* strains, based on a 548 bp long sequence section of their *tuf* gene a Neighbour-joining tree was constructed. The identified strains appeared next to their reference strains. The unidentified strains positioned near to their most closely related species. Unidentified strains #6 and #7 positioned in the group of *M. terrae* complex including *M. nonchromogenicum* as the most closely related species to the two unidentified isolates, while unidentified strain #4 clustered as an outgroup of the group composed of species such as *M. vanbaalenii, M. vaccae* and *M. gilvum*. Unidentified strain #3 had similar sequence to *M. nebraskense*, while *M. sp.* unidentified #5 to *M. colombiense*. Unidentified wild boar strains #1, #2, and #8 located at distant parts of the phylogenetic tree and had the highest sequence similarity to *M. thermoresistible, M. florentium* and *M. europaeum*, respectively.

Genotyping of *M. avium* subspecies

Large sequence polymorphism LSP^A17 was tested in all 962 *M. avium* strains. LSP^A17 was present in all MAP strains, while was missing from all MAA and MAS strains. It was present in 67.1% (64) of the MAH strains.

Beside 3 type strains MIRU−VNTR analysis was performed on 795 strains. Full profile was generated for 772 strains (135 MAA, 62 MAS, 84 MAH, 491 MAP).

The highest allelic diversity was observed at locus MIRU3 in MAA and MAH strains, MIRU2 in MAP strains, while in MAS strains MIRU4 was found to be the most polymorphic. Only one TR type was observed in MAP strains at MIRU4, MAA strains at MIRU1 and VNTR25, and in MAS strains at MIRU1, MIRU2, VNTR25, VNTR32 and MATR9 loci.

Within the 772 strains 85 different genotypes were detected. The described genotypes did not display host species preference nor differed in their prevalence throughout the years. Expect for genotypes 68 and 70, which seem to be present only in certain regions of Hungary, the occurrence of the genotypes showed no territorial differences.

The detected genotypes showed subspecies specificity. Within MAA strains 17, MAH strains 43, MAS strains 6 and MAP strains 19 different genotypes were observed.

MAA strains displayed the highest diversity in chickens, while MAH strains in wild boars.

Within MAP subspecies two dominant genotypes were found, the 77 and 84. Apart from a few piling in domestic and wild animals certain genotypes occurred in approximately the same proportion.

In certain farms the same genotypes were isolated during different epidemics, separated by long time-lapses. In single epidemics MAA strains always had the same genotype, while MAH strains belonged to different ones. The MAP strains from cattle (455) originated from 150 different farms. Family relations existed among the MAP hosts in six herds. The related animals had the same genotype in 10 cases out of 14.

The incidence of the two main MAP genotypes was the same in wild animals and cattle, but within cattle the different cattle types displayed different dominant genotypes. Within beef cattle the 84, while in dairy cattle the 77 was dominant. In the native Hungarian breeds (Hungarian Grey Cattle, and Hungarian Piebald) also genotype 84 was dominant. Dairy cattle (Jersey and Brown Swiss) imported mainly from Denmark and Germany and from Canada and the USA (Holstein-Friesian) beared almost exclusively genotype 77, while beef cattle from Slovakia and France had genotype 84.

On the philogenetic tree, representing the evolutionary history of the genotypes, the MAH, MAA, MAS and MAP strains arranged in distinct groups. MAH strains formed 3 clusters, while MAA strains not only had smaller and larger clusters, but independent genotypes also. MAS strains formed one cluster except one strain. Within MAP strains the type I strains formed a

separate branch, while within type II strains beside clusters independent genotypes were also observed.

The DI generated for all four subspecies was 0.9175, while determined for the subspecies separately, it resulted in 0.8679 for MAA, 0.9762 for MAH, 0.3419 for MAS and 0.7159 for MAP strains. DI within birds, domestic animals and wild animals was 0.967, 0.8583 and 0.9601 respectively.

Strain collection

From the strains isolated between 2006 and 2015 a strain collection (HUN_MYCO_COLLECTION_2006-2015) was created. One part (A) of the collection contains the 38 strains which were isolated from mastitic cattle milk samples. The other part (B) contains the strains which were not isolated from milk samples (2417: 541 MTC members, 972 MA, 904 NTM isolated from animals or environmental samples), along with reference strains and strains from ring test studies and of human origin. Beside data of the herds and host animals (age, year of birth, breed, sex, production type) we also recorded the type of the sample (lymph node, gut, faeces, etc.), its arrival time to the laboratory, the aim of the test (in case of positive or inconclusive tuberculin skin test results: diagnostic, without any pathological changes or in case of negative skin test results: monitoring), the gross pathological findings and histological results. For each strain we recorded the results of identification and typing tests carried out in the course of this study and the storage location where the strain is preserved.

Discussion

The almost worldwide occurrence of positive tuberculin skin test reactions in cattle highlight, that NTM can sensitise the host organisms, thus making the *in vivo* diagnostic of bovine tuberculosis difficult. Although the European Union recognized the tuberculosis free status of the Hungarian cattle farms in February 2014, wild animals can furthermore maintain and spread *M. caprae*, just like NTM, which emphasizes their role. Since the majority of NTM has proven to have zoonotic potential, so infected animals pose a threat to the carers, the veterinarians, hunters, as well as slaughterhouse and meat factory workers.

A key objective of our study was to identify non *M. avium* NTM by molecular biological methods, and to evaluate their occurrence and diversity. Our results show, that non *M. avium* NTM occur in great numbers among domestic and wild animals in Hungary, whose clinical significance can only be assessed after accurate identification. For the identification of non *M. avium* NTM from veterinary samples, the resolution of *tuf* and *rpo*B genes proved to be appropriate.

Commercially available kits were only able to identify a narrow range of the strains. These strains were of human health significance, which naturally does not coincide with species judged to be important in the field of animal health. Despite the use of putatively unique labeled DNA sequences, the specificity of these systems is not satisfactory. On the veterinary sample collection tested in the course of our study, very low (INNO-LiPA Mycobacteria v2: 10% Genotype Mycobacterium CM / AS 22%) identification rate was observed.

In our study, we identified 31 previously described non *M. avium* NTM species, from the environmental occurence, pathogenecity and zoonotic potential of which we already had some information. Unidentifiable strains were found in 14 cases. The diversity of environmental non *M. avium* NTM is much higher as it was known in the early 1990s. While in cattle samples only one unidentified strain type was found, in wild animal samples all 8 types were present. NTM species are taken up mostly from the environment. Since wild animals live in closer connection to their natural environment (soil, natural waters, sediments, plants), this may explain the diversity of the new strain types among them.

The new strain types were isolated usually only in single cases, expect *M. sp.* #4 strain, which we found five times. Besides three beef cattle, which reacted in tuberculin skin testing, it was also isolated from two red deer samples. This proves on one hand the constrant presence of the strain in the environment, and on the other hand, that it is able to cause infection and immunological sensitisation.

Mycobacterium species present in the environment can be collected, carried and transmitted through wild animals. Our observation, that the new strain types usually do not cause any pathological changes, also highlights the reservoir role of the host species.

In wild animals and cattle we found almost the same number of non *M. avium* NTM strains, but the panel of the strains was different, which imlies differences in the host species preference and pathogenecity of the strains.

Another objective of our study was to identify *M. avium* strains by molecular biological methods to the subspecies level and to investigate their genetic diversity.

MAA is the pathogen of avian tuberculosis, which is clearly indicated by the fact, that from birds we almost exclusively isolated this subspecies from typical pathological changes. Besides birds we also isolated it from other species, similarly to earlier findings, however its presence in red fox was reported first. The sensitivity of the different hosts to this pathogen is indicated by the proportion of pathological changes in infected animals. The gross pathological lesions observed in swine in high proportions and in cattle in low numbers are consistent with literature data. However, the dominance of this subspecies among swine is contradictory to previous findings, as earlier MAH subspecies was isolated more frequently from domestic pigs.

The ubiquity and broad host species spectrum of MAH strains was also known. Contrary to MAA, MAH is rarely isolated from birds. In our study the two strains were isolated from zoo birds, which could get infected from a drinker, where MAH strains easily form biofilms. The MAH infection of the dog was particularly interesting, as carnivores are usually resistent against *M. avium* infections. However, some authors suppose the presence of genetic susceptibility in certain breeds, just like the miniature Schnautzer in our case. The occurrence of MAA and MAH strains in cattle showed no difference regarding the production type, age or sex of the animals.

MAP strains have already been isolated from a wide range of animals. Wild animals, such as foxes or wild boars are known reservoirs of the pathogen.

Our isolates belonged to type II expect 3 sheep, 3 goat and one cattle strains. Although in cattle type II is the most common, we also showed, that type I can also occur. At the same time the broad host species spectrum of type II strains was also proved, as it was isolated from 8 different species. These results are consistent with previous findings; expect that the swine strain also proved to be type II contrary to previous results. Besides, we also isolated type II strains from goats. To our knowledge, we isolated MAP strains from swine, wild boars, red fox, red deer, mouflon and buffalo for the first time in Hungary.

The origin of the isolated strains corresponds to the lifestock breeding and wildlife distribution of Hungary. The sheep isolates came from the northern part of the Great Plain where the small ruminant breeding is significant, while the wild boar, red deer and red fox strains originated from the region of the Transdanubian Mountains known for its rich wildlife populations. More than 85% of the cattle hosts from which MAP was isolated as subsidiary findings reacted in tuberculin skin testing, which confirms that MAP is able to cause immunological sensitisation in the hosts.

The results of targeted detection of MAP by direct PCR analysis show that removal of PCR inhibitory substances is necessary in case of faecal samples.

MAS strains have previously only been isolated from deer beside birds, and by cattle only artificial infection was published. Although Turenne has questioned the authenticity of the subspecies in a review article in 2007, the number of strains isolated in recent years demonstrates its significance.

We isolated MAS strains for the first time from red fox, red deer, wild boar, cattle and badger. Since this subspecies requires special culture conditions, its isolation requires particular attention and experience. When in 1990 it was classified as a subspecies of *M. avium* its identification was possible only on the basis of phenotypic characteristics. During the past decades numerous attempts were made for its molecular biological identification but due to the high degree of sequence similarity with the MAA strains, they failed. Our assay enables the accurate and reliable identification of MAS strains and their discrimination from MAA strains. As this method does not require probes, labeled primers, or any post-PCR processing, it is a rapid and cost-effective identification method. Parallel gene scanning in a single tube strengthens the trustworthiness of the method. The stable T_m differences of the HRM curves and the low standard deviation of the T_m of the MAS-specific amplification products contribute to the robustness of the system, while the wide input DNA range makes it flexible and ready to use for routine diagnostic laboratories.

Investigations on the genetic diversity of the strains are essential for the understanding of the epidemiological differences of the strains and the origin of the different infections.

LSPs are molecular markers of the genetic diversity within MTC and *M. avium* strains. The results of our LSP^A17 sequence polimorphism tests are consistent with previous data.

MIRU-VNTR analysis for typing and investigation of the genetic diversity of *M. avium* strains was first used in 2007. Since then, numerous new loci were identified and tested on different panels of *M. avium* strains in various combinations. In order to achieve a higher DI, a unique loci set was applied including 4 MIRU, 3 VNTR and one MATR loci. Alltogether MIRU3 proved to be the most diverse, however among MAP strains MIRU2, while among MAS strains MIRU4 was the most variable. As the variability of the loci is different among the subspecies, by planning a future study this should be considered.

The subspecies specificity of the different genotypes is consistent with previous results. Our results demonstrate the high diversity of MAH strains and the relative uniformity of MAS strains.

The widespread occurrence and constant presence of the different genotypes proves their extreme environmental tolerance. The presence of genotypes at the same area in different animal species confirms the transfer between species, or the possibility of infection from the same source of infection.

Our finding, that concurrent MAA infections are caused by the same genotype, while MAH infections by different genotypes, hypothesises that MAA and MAH strains differ in their primary route of transmission. In case of MAH route of infection seems to be environmental. On the contrary, as MAA strains are less viable in the environment, in this subspecies disease transmission from one infected animal to another appears to be dominant.

In case of MAP strains the relatively high proportion of farms with multiple profiles is attributable to frequent cattle exchange. The infected animals introduced into a new herd not only increase the MAP environmental impact, but also can directly transfer the pathogen to companion animals and their calves, as evidenced by the observed large-scale genotype agreement between related animals.

Although small number of isolates originated from imported animals, we made an interesting observation. The imported beef and milk cattle consistently carried different genotypes independently of their breed or country of origin. At the same time, the native Hungarian breeds also shared one distinct genotype. We hypothesise, that the production type of the animals is somehow connected to their MAP genotype. After the 1970s, the native double use cattle population in Hungary was progressively replaced with Holstein-Friesians (mainly from Canada and the USA). Although retrospective studies could not be performed, we hypothesise that the Holstein-Friesian breed could have brought along genotype 77, which has spread and probably repressed the native genotype 84. This theory is only an assumption, which in absence of retrospective analyzes might not even be proven, but it definitely raises the possibility that the occurrence of the different genotypes and the possibility of infections caused by them, can be related to environmental factors or genetic properties of the host animals.

MAS infection in cattle has only been reported after artificial infection. In this study the same MAS genotype was detected in grazing beef cattle at two different farms. The detected genotype was the most prevalent among red deer and wild boars of the same region. This observation could point to the same source of infection, would suggest interspecies disease transmission or could be the result of permanent and widespread environmental presence of the genotype. Our observation, that wild animals reflect the overall genotype proportions is also coherent with their role as reservoirs contributing to spread and maintenance of the diseases. Sylvatic cycles of *Mycobacterium* strains may not only undermine control and eradication programmes but also facilitate farm-to-farm spread of the disease. All these observations highlight the need to improved epidemiological control measures, in order to prevent the spread of the infection between farms and reservoir species.

In the current study, the distribution of the different genotypes on the NJ tree is in accordance with the genetic relationship among the subspecies.

The loci set used enabled the increase of discriminatory power compared to previous loci combinations, thus can be recommended for future studies.

Despite the long-standing Mycobacterium diagnostics in Hungary, our investigations are gap filling. Our results greatly contribute to the expansion of our knowledge about the occurrence, pathogenicity, and host spectrum of NTM in Hungary. The strain collection created in this study provides excellent basis for further investigations.

New Scientific Results

Overall: we renewed and placed the Hungarian veterinary *Mycobacterium* diagnostic on molecular biological basis. From the results of this work I would highlight the following:

Regarding *M. avium* and its subspecies:

1. In Hungary we identified the subspecies of *M. avium* for the first time by molecular biological methods, and provided data on their occurrence, host species spectrum and pathogenicity.

2. We developed new media for the isolation of MAS strains, and developed a HRM and MM duplex real-time PCR assay for the specific identification of the strain.

3. We isolated *M. avium* subsp. *silvaticum* strains for the first time from red fox, red deer, wild boar, cattle, and badger, type II *M. avium* subsp. *paratuberculosis* strains from swine, and *M. avium* subsp. *avium* from red fox.

4. In Hungary we identified the types of *M. avium* subsp. *paratuberculosis* strains for the first time and revealed the presence of type I strains in cattle, goat and sheep. We introduced new media for the isolation of type I strains.

5. In Hungary we tested the *M. avium* subspecies for the first time for the presence of $LSP^{A}17$ and with MIRU−VNTR.

Regarding the non *M. avium* NTM:

6. In Hungary we identified for the first time non *M. avium* NTM by molecular biological methods.

7. Among samples from wild, domestic and pet animals we detected 31 known NTM species and 8 new strain types, and provided data on their occurrence, host species spectrum and pathogenicity: some species or subspecies were isolated from new hosts, and we also found gross pathologic lesions in infections caused by species, which were not considered to be pathogenic before.

8. From the isolated and type stranis a strain collection was created containing 2481 strains with detailed data of their origin.

Scientific Publications

Jánosi, Sz., Rónai, Zs., Dán, Á., Glávits, R., Barta, E.: „Egzotikus" kórokozók okozta tőgygyulladások Magyarországon: *Prototheca,* gombák, *Corynebacteriumok* és *Mycobacteriumok*, MÁL, 134: 15−20, 2012. IF(2012): 0,146

Bende, B., Jakab, Cs., Balka, Gy., Rónai, Zs., Jánosi, Sz., Vajdovich, P., Biksi, I.: Szisztémás *Mycobacterium avium* subsp. *hominissuis* fertőzés törpe schnauzerben, MÁL, 135: 138−148, 2013. IF(2013): 0,185

Ács, K., Rónai, Zs., Nagy, G., Csivincsik, Á., Sugár, L., Jánosi, Sz.: *Mycobacterium caprae* és *Trueperella (Arcanobacterium) pyogenes* társfertőzés okozta tályogképződés dámszarvas (*Dama dama*) májában és májkapui nyirokcsomóiban – Esetismertetés, MÁL, 136: 618−621, 2014. IF(2014): 0,185

Csivincsik, Á., Rónai, Zs., Nagy, G., Varga, Gy., Jánosi, Sz.: Új szemléletmód a szarvasmarha-gümőkór mint széles gazdaspektrumú fertőző betegség járványtanában, MÁL, 136: 631−639, 2014. IF(2014): 0,185

Rónai, Z., Csivincsik, Á., Gyuranecz, M., Kreizinger, Z., Dán, Á., Jánosi, S.: Molecular analysis and MIRU-VNTR typing of *Mycobacterium avium* subsp. *paratuberculosis* strains from various sources, J. Appl. Microbiol., 118(2): 275−283, 2015. IF(2014*): 2,479

Rónai, Zs., Csivincsik, Á., Szőgyényi, Zs., Bacsadi, Á., Dán, Á., Jánosi, Sz.: Adatok a paratuberkulózis hazai előfordulásáról – diagnosztikai fejlesztések és vizsgálati eredmények, 2006–2012, MÁL, 137: 211−218, 2015. IF(2015): 0,212

Rónai, Z., Csivincsik, Á., Dán, Á.: Molecular identification of *Mycobacterium avium* subsp. *silvaticum* by duplex high-resolution melt analysis and subspecies-specific real-time PCR, J. Clin. Microbiol., 53(5): 1582−1587, 2015. IF(2014*): 3,993

Rónai, Zs., Csivincsik, Á., Gyuris, É., Rigó, D., Dán, Á.: *Mycobacterium avium* subspecies *avium*, „*hominissuis*" és *silvaticum* alfajok jelentősége és magyarországi előfordulása, MÁL, 137: 549−556, 2015. IF(2015): 0,212

Rónai, Z., Csivincsik, Á., Dán, Á., Gyuranecz, M.: Molecular analysis and MIRU-VNTR typing of *Mycobacterium avium* subsp. avium, '*hominissuis*' and *silvaticum* strains of veterinary origin, Infect. Genet. Evol., 7(40): 192−199, 2016. IF(2014*): 3,015

Rónai, Z., Eszterbauer, E., Csivincsik, Á., Guti, Cs., Dencső, L., Jánosi, Sz., Dán, Á.: Detection of wide genetic diversity and several novel strains among non-avium non-tuberculous *Mycobacteria* isolated from farmed and wild animals in Hungary, J. Appl. Microbiol., 121(1): 41−54, 2016. IF(2014*): 2,479

Csivincsik, Á., Rónai, Zs., Nagy, G., Nagy, E., Sugár, L.: A vadállományban előforduló szarvasmarha-gümőkór jelentette járványtani kockázat kezelése igazgatási eszközökkel, MÁL, 138: 209−217, 2016. IF(2015*): 0,212

Csivincsik, Á., Rónai, Z., Nagy, G., Svéda, G., Halász, T.: Surveillance of *Mycobacterium caprae* infection in a wild boar (*Sus scrofa*) population in south western Hungary, Vet. Arhiv, 86: 767−775, 2016. IF(2015*): 0,321

Csivincsik, Á., Rónai, Zs., Szabó, J., Lővey, L., Jánosi, Sz.: Environmental epidemiology of bovine tuberculosis in pastured cattle and deer herds, https://www.landwirtschaft.sachsen.de/landwirtschaft

Eszterbauer, E., Rónai, Zs., Marton, Sz., Ursu, K., Baska, F., Láng, M.: *Mycobacterium* fajok terjedése kereskedelmi forgalomban kapható fagyasztott haleleségek útján, Halászatfejlesztés, 34: 71−77, 2012.

Csivincsik, Á., Rónai, Z., Nagy, G.: Post mortem examination of submandibular lymph node in wild boars (Sus scrofa) as a beneficial part of bovine tuberculosis surveillance systems, Acta Agraria Kaposváriensis, 19: 8−12, 2015.

Conference presentations

Jánosi, Sz., Dencső, L., Dán, Á., Rónai, Zs.: *Mycobacterium caprae* hazai előfordulása 2006-ban és 2007-ben, Akadémiai Beszámolók – előadás, 2008.

Jánosi, Sz., Rónai, Zs., Dán, Á., Dencső, L.: Atípusos mycobacteriumok előfordulása szarvasmarha szervmintákban és telepi környezetben, Akadémiai Beszámolók – előadás, 2008.

Csivincsik, Á., Rónai, Zs., Jánosi, Sz., Szabó, J.: A vaddisznó fertőzésfenntartó szerepe a szarvasmarha-gümőkór járványtanában a Zselicben, Akadémiai Beszámolók – előadás, 2009.

Rónai, Zs., Csivincsik, Á., Dán, Á., Dencső, L., Jánosi, Sz.: Újabb adatok a „paraallergiás" reakciókról, különös tekintettel a hazai szarvasmarha-állományok paratuberculosisára, valamint atípusos *Mycobacterium* fertőzéseire, Magyar Buiatrikusok Társaságának XIX. Nemzetközi Kongresszusa – előadás, 2009.

Jánosi, Sz., Csivincsik, Á., Rónai, Zs., Aranaz, A., Rodriguez, S., de Juan, L., Pálfalvi, A.: Tuberculosis szarvasmarhában és más állatfajokban Magyarországon: aktualitások és humán-egészségügyi kockázatok, Szent-Iványi – Binder napok, Rudnai-Kemenes Tudományos ülés – előadás, 2009.

Rónai, Zs., Dán, Á., Dencső, L., Jánosi, Sz.: A hazai szarvasmarha-állományok atípusos Mycobacterium fertőzései, a diagnosztika nehézségei, Akadémiai Beszámolók – előadás, 2010.

Rigó, D., Rónai, Zs., Erdélyi, K.: Egzotikus madarak kórbonctani labordiagnosztikája, Magyar Vad- és Állatkerti Állatorvosok Társaságának és a Fővárosi Állat- és Növénykert közös konferenciája – előadás, 2010.

Rónai, Zs., Csivincsik, Á., Dán, Á., Jánosi, Sz.: Non-tuberculous Mycobacteria in animals in Hungary, 31th Annual Congress of the European Society of Mycobacteriology – poszter, 2010.

Csivincsik, Á., Nemes, Cs., Rónai, Zs., Szabó, J., Jánosi, Sz.: Gümőkórra gyanút keltő elváltozások előfordulási aránya vaddisznókban a 2008-2010. közötti időszakban, a Zselicben, Akadémiai Beszámolók – előadás, 2011.

Rónai, Zs., Lami, E., Csivincsik, Á., Stollár, K., Dénes, B., Dán, Á., Jánosi, Sz.: A paratuberculosis fertőzöttség kimutatására végzett vizsgálatok tapasztalatai, Akadémiai Beszámolók – előadás, 2011.

Jánosi, Sz., Rónai, Zs., Dán, Á., Glávits, R., Barta, E.: Egzotikus kórokozók okozta tőgygyulladások Magyarországon: *Prototheca*, gombák, *Corynebacteriumok* és *Mycobacteriumok*, Magyar Buiatrikus Társaság 21. Nemzetközi Kongresszusa – előadás, 2011.

Rónai, Zs., Csivincsik, Á., Dán, Á., Jánosi, Sz.: Molecular identification of Hungarian *Mycobacterium Avium* Complex isolates of animal origin, 32th Annual Congress of the European Society of Mycobacteriology – poszter, 2011.

Eszterbauer, E., Rónai, Zs., Marton, Sz., Ursu, K., Baska, F., Láng, M.: Dissemination of *Mycobacterium spp.* via commercial fish food: Mycobacterium chelonae infects Silver carp, European Association of Fish Pathologists15th Conference – poszter, 2011.

Rónai, Zs., Dán, Á., Dencső, L., Csivincsik, Á., Jánosi, Sz.: Molecular Methods for the Identification of Mycobacterium Isolates with Animal Origin, Magyar Mikrobiológiai Társaság 16. Nemzetközi Kongresszusa – poszter, 2011.

Eszterbauer, E., Rónai, Zs., Marton, Sz., Ursu, K., Baska, F., Láng, M.: *Mycobacterium* fajok terjedése kereskedelmi forgalomban kapható fagyasztott haleleségek útján, Halászati Tudományos Tanácskozás, Haki napok – poszter, 2011.

Csivincsik, Á., Nagy, G., Svéda, G., Rónai, Zs., Jánosi, Sz.: Gümőkórra gyanút keltő kórbonctani elváltozások vaddisznó-populáción belüli előfordulási arányával összefüggő kockázati tényezők elemzése, Akadémiai Beszámolók – előadás, 2012.

Jánosi, Sz., Rónai, Zs., Barta, E., Dénes, B., Lami, E., Dán, Á., Szeredi, L., Erdélyi, K., Gyuranecz, M., Makrai, L., Csivincsik, Á.: A sertés brucellózis és gümőkór aktuális kérdései, Köves napok – előadás, 2012.

Jánosi, Sz., Csivincsik, Á., Rónai, Zs., Rodriguez, S., de Juan, L., Aranaz, A.: Tuberculosis and other mycobacterial infections of wildlife in Hungary, 9th Biennial Conference of the European Wildlife Disease Association – poszter, 2012.

Csivincsik, Á., Nagy, G., Balog, T., Varga, Gy., Rónai, Zs., Jánosi, Sz.: A populáció-biológiai változások és a gümőkór kórbonctani prevalenciájának összefüggései vaddisznóban, Akadémiai Beszámolók – előadás, 2014.

Rónai, Zs., Dán, Á., Csivincsik, Á., Gyuranecz, M.: Comparative MIRU-VNTR analysis of *Mycobacterium avium* subspecies isolates of veteriary origin, Magyar Mikrobiológiai Társaság 17. Nemzetközi Kongresszusa – poszter, 2015.

Rónai, Zs., Csivincsik, Á., Gyuranecz, M., Kreizinger, Zs., Szőgyényi, Zs., Dán, Á., Jánosi, Sz.: *Mycobacterium avium* ssp. *paratuberculosis* törzsek hazai elterjedtsége és MIRU-VNTR elemzése, Akadémiai Beszámolók – előadás, 2015.

Csivincsik, Á., Rónai, Zs., Nagy, G.: "A szarvas az más" - állathigiéniai kihívások és lehetséges megoldásuk az alternatív gazdasági állatfajok tartása során, Magyar Buiatrikus Társaság 26. Nemzetközi Kongresszusa: In Memoriam Kovács Ferenc Nemzetközi Állatorvos és Állattenyésztő Kongresszus. 375 p – 239−242., 2016.

Csivincsik, Á., Rónai, Zs., Nagy, G.: One Health approach in free-ranging systems – bovine tuberculosis as a model, 24th Int. Symp. “Animal Science Days”. Ptuj, Szlovénia, Acta Agriculturae Slovenica Supplement 5: 28−30., 2016.

Csivincsik, Á., Rónai, Zs., Nagy, G.: Potential target species for surveillance on a bovine tuberculosis endemic area, VII International Scientific Agriculture Symposium "Agrosym 2016". Jahorina, Bosznia-Hercegovina, Book of abstracts. 1226 p – 972., 2016.

Acknowledgements

I owe thanks to Prof. Dr. Péter Rudas and Prof. Dr. Gyula Huszenicza, who embarked me on this way.

First I would like to thank my supervisor, Dr. Ádám Dán for his patience, support and help.

I owe thanks to the leaders and coworkers of our Doctoral School, just as the former (Dr. Lajos Tekes) and current leader (Dr. Tamás Abonyi) of my workplace.

Special thanks to the academic staff, co-workers and assistants of the department of Bacteriology and Molecular Biology for their help and support, especially to Ildikó Szombatiné Boda, Sándorné Nagy, Ernőné Ottinger, Ágnes Juhász and Dr. Krisztina Ursu.

I thank Erzsébet Köles and the coworkers of the University Library for their help in collecting the literature.

Special thanks are granted for Julcsi Turák, Andris Mocsári, Dóri Horváth, Kinga Tarjániné Szabó, Annamária Surján, Jánosné Veress and Tímea Miskó for their endless helpfulness and support.

I also would like to thank colleaques at other departments of my workplace: Ákos Thuma, Éva Gyuris, Dóra Rigó, Károly Erdélyi, Gergő Tóth, Árpád Bacsady and Endre Bajmócy.

I am thankful to Miklós Gyuranecz, Zsuzsa Kreiczinger and Edit Eszterbauer for their help in revising my publications.

I owe thanks to my friends (Nóra Schweitzer, Bernadett Khayer, Márta Lőrincz, Ama Szmolka), who undertook to read through my Doctoral Thesis with a critical eye.

Special thanks to Ági Csivincsik for the samples, information, her support and encouragement.

Finally, I would like to thank my previous and current colleaques, PhD classmates, my friends and my family for their patience and encouragement, and for my Mother, that she always believed in me.