Strategies for differentiation, identification and typing of medically important species of mycobacteria by molecular methods

L. Dvorská[1], M. Bartoš[1], G. Martin[2], W. Erler[2], I. Pavlík[1]

[1]*Veterinary Research Institute, Brno, Czech Republic*
[2]*Federal Institute for Health Protection of Consumers and Veterinary Medicine, Department Jena, Germany*

ABSTRACT: Molecular biology methods offer new opportunities to differentiate, identify and type bacterial species and strains. These methods use the variability of nucleic sequences of genes such as 16S rDNA**,** beta subunit RNA-ase (*rpo*B), gyrase (*gyr*B), rDNA internal transcribed spacer and other genes. The aim of this paper is to provide comprehensive information about the methods available to differentiate and identify species of mycobacteria at the DNA sequence level. The methods discussed in the review include PCR, PCR-REA, sequencing analysis, spoligotyping and DNA fingerprinting. These methods have been applied to both the "universal" part of the genome and to specific mycobacterial genes.

Keywords: human, bovine and avian tuberculosis; paratuberculosis; avian mycobacteriosis, Johne's diseases; 16S rDNA; internal transcribed spacer 16S-23S rDNA; insertion sequence; PCR; PCR-REA; RFLP; sequencing analysis, spoligotyping, repeat sequence

Abbreviations: ***aph*C** – gene encoding alkylhydroxyperoxidase C; **ATCC –** American Type Culture Collection; **BCG** – *Bacillus* Calmette-Guérin; **bp** – base pair; **CNCTC** – The Czechoslovak National Collection of Type Cultures; **CPM** – conditionally pathogenic mycobacteria; **BLAST** – Basic Local Alignment Search Tool; **DNA** – deoxyribonucleic acid; ***dna*J** – gene encoding cold-shock protein DnaJ; **DR** – direct repeat; **EMB** – Etambutol; **ETR** – exact tandem repeats; **FR** – flanking region; ***gyr*B** – gene encoding beta subunit of gyrase; **IS** – insertion sequence; **ITS** – rDNA internal transcribed spacer; **INH** – Isoniazid; **HIV/AIDS** – Human Immune-Deficiency Virus/Acquired Immune Deficiency Syndrome; **HPA** – hybridisation protection assay; ***hsp*** – heat shock protein; ***kat*G** – gene encoding catalase G; **kDa** – kilo Dalton; **LCR** – Ligase Chain Reaction; ***M.*** – *Mycobacterium*; ***MAC*** – *M. avium* complex; **MDR–*MTB*** – multi-drug-resistant strains of *M. tuberculosis*; **MPTR** – major polymorphic tandem repeat; ***MTC*** – *M. tuberculosis* complex; ***mtp*40** – gene encoding protein of *M. tuberculosis*; **NBT** – α-naphthyl butyrate; **ORF** – open reading frame; ***oxy*R** – gene encoding protein of oxidative stress response; **PCR** – polymerase chain reaction; **PCR–REA = PRA** – polymerase restriction analysis; **PGRS** – polymorphic GC–rich repeat sequence; **PE** – proteins with motifs of amino acids Pro-Glu; ***pnc*A** – gene encoding pyrazinamidase A; **PPE** – proteins with motifs of amino acid Pro-Pro-Glu; **PZA** – Pyrazinamide; **RNA** – ribonucleic acid; **rRNA** – ribosomal RNA; **rDNA** – ribosomal DNA; **RE** – restriction endonuclease; **REA** – restriction endonuclease analysis; ***rec*A** – gene encoding recombinase A; **RFP** – Rifampicin; **RFLP** – restriction fragment length polymorphism; ***rpo*B** – gene encoding beta subunit RNA-ase; **SDA** – strand displacement amplification; **spoligotyping** – spacer oligotyping; **Sp.** – species; **Subsp**. – subspecies; **STM** – Streptomycin; **TMA** – transcription mediated amplification; **VNTR** – variable numbers of tandem repeats.

CONTENTS

Partially supported by the Ministry of Agriculture of the Czech Republic (Grants No. QC0195 and No. MZe-M03-99-01), EC project SACROHN (No. QLK2-CT-2000-00928) and the Ministry of Education, Youth and Sports of the Czech Republic (No. ME473).

1. INTRODUCTION

Even at the beginning of the twenty-first century, mycobacterial infections in man remain a serious medical problem world-wide (Thoen and Steele, 1995; Grange, 1996; Kubín, 1996). Apart from strains of the *Mycobacterium tuberculosis* complex (*MTC*), which cause tuberculosis, atypical mycobacteria, otherwise called conditionally pathogenic mycobacteria (CPM) can cause human infections (mycobacterioses). The importance of CPM has been increasing in the last two decades. *M. avium* complex (*MAC*), *M. kansasii, M. xenopi, M. fortuitum* and also other species are frequent causes of disease. The main reason for the dramatic increase in infection caused by these species of mycobacteria is the current world-wide HIV/AIDS pandemia. The clinical symptomatology of these diseases is no different from classical tuberculosis (Böttger, 1995; Grange, 1996; Bártů, 1998).

The basis for the unambiguous diagnosis of mycobacterial infection and subsequent effective treatment is the isolation and identification of the bacterial species. Because of the long generation time of mycobacteria, standard cultivation and biochemical methods used to establish phenotypic characteristics are time-consuming. Moreover, the phenotype of mycobacterial cultures is not stable, but demonstrates a striking variability, depending on the cultivation conditions (Kirschner and Böttger, 1998). Methods based on lipid analysis (gas chromatography, highly effective liquid chromatography or thin-layer chromatography) are technically demanding and expensive and are therefore only used in a few specialised laboratories.

Fast, easy and sensitive methods based on the detection of genetic diversities have been developed in increasing numbers in the last decades and are now available for differentiation and identification of individual mycobacterial species and typing of individual strains for epidemiological analyses. The terms differentiation, identification and typing are often used with different sense in the literature. In this paper we use the following definitions:

1. **differentiation** – method to distinguish between several species, example is the sequencing of the 16S rDNA,
2. **identification** – one specific form of differentiation, directed to a particular group of species, individual species or subtypes, such as the detection of *MTC* by the PCR amplification of IS*6110* or the detection of *M. a. paratuberculosis* by PCR amplification of IS*900*,
3. **typing** – method for detailed description of individual strains within a species which is necessary for epidemiological analysis and tracing of outbreak strains, examples are PCR-RFLP methods based on specific IS elements, which are used for several species.

The aim of this paper is to provide comprehensive information about the methods available for the differentiation and identification of mycobacterial species at the DNA level, which will be increasingly important for medical and veterinary laboratories in the future. This work extends previous overviews: McFadden *et al*. (1987, Kirschner and Böttger (1998), Kremer *et al*. (1999) and Algorithm for molecular differentiation of mycobacteria (Dostal *et al*., 2001, http://www.ridom.de/mycobacteria/).

2. COMMERCIAL MOLECULAR METHODS FOR THE IDENTIFICATION OF MEDICAL IMPORTANT SPECIES OF MYCOBACTERIA

Two strategies have been developed into commercially available test systems for differentiation and identification of mycobacteria. Both strategies rely on an initial nucleic acid amplification step. The systems differ in the

detection of the amplicon. In the direct detection systems the amplicon is detected in biochemical reactions with species specific DNA probes. In contrast, the second group of the tests is based on a specific reverse hybridisation step of the amplification product against fixed DNA of different mycobacteria species on a blotting membrane.

Following the first strategy, four commercial test systems are available. Transcription Mediated Amplification (TMA) and Hybridisation Protection Assay (HPA) by Gen-Probe Incorporated, Ligase Chain Reaction (LCR) followed by an antibody-antigen reaction to detect the ligated probe (Abbott Laboratories), PCR and a biotin-avidin horseradish peroxidase detection system (Roche Diagnoistics) and the Strand Displacement Amplification (SDA) and homologous real-time detection system (Becton Dickinson Probe TecET). All systems of this group of tests are focused on a limited number of mycobacterial species, for example, the Accu-Probe system has been designed to identify *M. tuberculosis, MAC*-species, *M. kansasii* and *M. gordonae* (Gen-Probe Incorporated, San Diego, California, USA).

The target of the reverse hybridisation assays (group two) is the 16S-23S rRNA spacer region which is amplified in a PCR-reaction with a biotinylated primer system. The amplification products are subsequently hybridised to typing strips onto which parallel DNA probe lines and control lines are fixed. After hybridisation streptavidin labelled with alkaline phosphate is added and bound to any biotinylated hybrid previously formed. Incubation with BCIP/NBT chromogen results in a purple/brown precipitate. The two commercially available systems differentiate 12 mycobacterial species and *MTC* (HAIN Diagnostica, Nehren, Germany) and 8 mycobacterial species and genus *Mycobacterium* (INNOGENETICS, Heiden, Germany), respectively. The performance of both tests is very good, and the fast majority of isolates was detected correctly. Both tests include a number of atypical mycobacteria species of clinical importance which are not covered by the other test systems (Boden *et al*., 1998; Tortoli *et al*., 2001).

2.1. Methods for differentiation between species using the 16S rDNA sequence

2.1.1. Amplification and sequencing analysis of the 16S rDNA gene

The 16S and 23S rRNA genes are particularly suitable as targets for identifying microorganisms down to the species level. With the exception of viruses, rRNA genes are found in all organisms. Ribosomal nucleic acids are considered to be phylogenetically meaningful molecules that provide a record of evolution. When organisms evolved into what we call domains, divisions, classes, families, genera, and species, these events were imprinted in the sequences of rRNA. These imprints, or molecular signatures, form the basis for identifying microorganisms (Kirschner and Böttger, 1998).

The sequence of the 16S rDNA gene is specific at the species level and is also a stable property of microorganisms. Hypervariable regions (between 129 bp – 276 bp and 430 bp – 495 bp) are particularly useful to resolve within-species variation. In one study (Kirschner and Böttger, 1998) so called "universal primers" UNB51 (5′-GAG TTT GAT CCT GGC TCA-3′) in position 8 bp to 27 bp, and UNB800 (5′-GGA CTA CCA GGG TAT CTA AT-3′) in the position 806 bp to 787 bp were designed on the basis of the *E. coli* 16S rDNA gene sequence. The resulting amplification product is 800 bp in size and may be reamplified using primers UNB51 and UNB52 (5′-ACC GCG GCT GCT GGC AC-3′; position 533 bp – 515 bp). The size of the reamplification product is 520 bp. UNB52 then serves as a primer for sequencing. By comparing the mycobacterial sequence obtained with the universal primers with published sequences of 16S rDNA using the BLAST database (Basic Local Alignment Search Tool) it is possible to reliably identify a particular bacterium. For example, the universal primers amplification method is particularly useful to resolve groups of *M. gordonae* which exhibits genome heterogeneity between the strains (Kirschner and Böttger, 1998). A disadvantage of this identification method is that the closely related species *M. kansasii* and *M. gastri* have identical 16S rDNA gene sequences (Kirschner and Böttger, 1998). A useful method to differentiate between these two species is to sequence the 16S-23S rDNA spacer region or to use the PRA method (Chapter 2.1.3.), described by Telenti *et al*. (1993).

2.1.2. Identification of *MAC*-species based on the 16S rDNA gene

Wilton and Cousins (1992) described a method for the simultaneous identification of genus, species and strains of *Mycobacterium* sp. using conserved and variable sequences of the 16S rDNA gene. By comparing 16S rDNA sequences of significant mycobacterial pathogens, they found variable regions specific for individual species. They used this information to develop a duplex amplification system, which makes it possible to identify the genus *Mycobacterium*, and the species *M. a. avium* and *M. intracellulare*. By combining the primers for 16S rDNA with primers specific to the gene which encodes the secretion protein MPB70 (specific for *MTC*), this system permits the detection and identification of clinically important mycobacteria in one single PCR.

Through PCR amplification of conserved regions of the 16S rDNA gene using genus-specific oligonucleotides MYCGEN-F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and MYCGEN-R (5′-TGC ACA CAG GCC ACA AGG

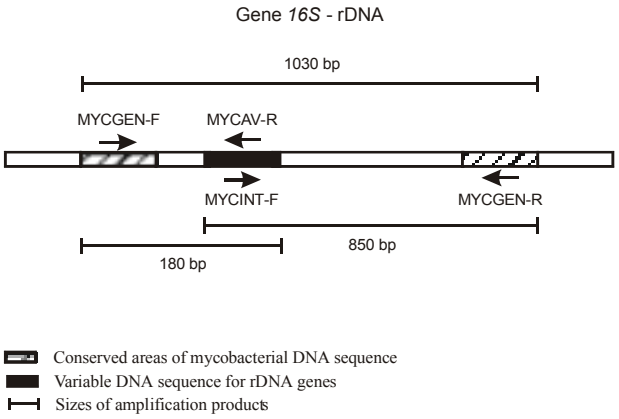

Figure 1. Annealing sites of genus- and species-specific primers on the 16S rDNA gene in *Mycobacterium* sp. (Wilton and Cousins, 1992)

GA-3′) which produce a 1 030 bp fragment, it is possible to identify the genus *Mycobacterium*. The combination of the external primer MYCGEN-F with the species-specific primer MYCAV-R (5′-ACC AGA AGA CAT GCG TCT TG-3′) results in a product of 180 bp which identifies the species *M. a. avium* and *M. a. paratuberculosis*. Using the combination of the primer MYCGEN-R with the species-specific primer MYCINT-F (5′-CCT TTA GGC GCA TGT CTT TA-3′), a 850 bp amplicon indicates *M. intracellulare* strains (Figure 1). A PCR system based on the above-mentioned primers can be used to identify non-virulent strains of *M. intracellulare*. The disadvantage of this method is that it does not distinguish between *M. a. avium* and *M. a. paratuberculosis*, which, on the other hand illustrates the high sequence homology in these two species.

2.1.3. Restriction profile analysis of the 16S rDNA gene

The PCR-REA (or PRA) method is based on the combination of PCR amplification of the 16S rDNA gene and subsequent restriction analysis. The primers MB-UZ1 (5′-GAC GAA CGC TGG CGG CGT GCT TAA C-3′) and MB-UZ2 (5′-CGT CCC AAT CGC CGA TC-3′) were derived from conserved regions of 16S rDNA by comparing 15 mycobacterial species (Thierry *et al*., 1990).

On the basis of sequence comparison using the programmes Gene Compar (Applied Maths, Version 4, Kortrijk, Belgium) and GCG (Wisconsin Package, Version 9.1, Genetics Computer Group, USA), we developed an identification system, which permits the differentiation of most clinical significant mycobacterial species in two steps. For amplification of the 16S rDNA gene primers according to Thierry *et al*. (1990) are used. The resulting PCR products, 1 300 bp in size, are digested using the REs *Rsa* I and *Cfo* I. From our sequence comparison results we concluded that with RE *Rsa* I we can differentiate between *M. tuberculosis* and *M. bovis* strains. In addition, with *Rsa* I it is possible to distinguish species of *MAC* (*M. a. avium* serotypes 1–3, 8–11 and 21, *M. a. paratuberculosis*) and *M. intracellulare* (serotypes 7, 12–20, 22–28). Because of the similarity in the size of restriction fragments of species *M. intracellulare, M. gordonae* and *M. ulcerans,* obtained after digestion by *Rsa* I, we also digest the 1 300 bp PCR product with the RE *Cfo* I. This method can be applied to differentiate the *M. ulcerans* species from the *M. terrae* species. Additionally, it is possible to differentiate closely related species from the genera *Corynebacterium*, *Rhodococcus*, *Gordonia* and *Nocardia* which often appear in samples together with mycobacteria. The system we developed does not permit the differentiation between *M. bovis* and *M. tuberculosis* subsp. *caprae*. Variability of the DNA sequence of serotypes especially in species of the *M. fortuitum* complex, and the serotypes of *M. kansasii* and *M. chelonae* also confounds their differentiation.

2.2. Sequencing analysis of the *rec*A gene

The *rec*A gene sequence is used as an alternative to the sequence analysis of the gene 16S rDNA for the differentiation of mycobacteria (Blackwood *et al*., 2000). Protein *Rec*A encoded by this gene exists in all bacteria. It has a role in homologous DNA recombination, DNA damage repair and induction of the SOS response. Two fragments, A and B, were gained through the amplification of *rec*A gene. Sequencing of the fragment A (915 bp – 970 bp) distinguished the species *M. leprae, M. aurum* and *M. mucogenicum* which indicated 75.7% similarity. Despite of 96% homology, the *rec*A sequencing can differentiate the clinically important *M. kansasii* from the less clinically important *M. gastri*. Sequencing of the fragment B (about 1 kbp) distinguished the species *M. xenopi, M. asiaticum, M. shimoidei* and *MTC*.

2.3. Sequencing analysis of the *rpo*B gene

The polymorphism of the *rpo*B gene, which encodes the beta subunit of RNA polymerase, was used to differentiate mycobacteria through DNA hybridisation and DNA sequence comparison (Lee *et al*., 2000). The variable region of *rpo*B in mycobacteria is suitable to be used in a PCR-REA assay. This variable region of the *rpo*B gene is flanked by conserved sequences. They enable the amplification of the variable region using the same pair of primers for all mycobacterial species. The *rpo*B region was amplified in 44 species of mycobacteria (Lee *et al*., 2000). The resulting amplification products, 360 bp in size, were subsequently digested using the REs *Msp* I and *Hae* III. Most of the mycobacterial species could be separated on the basis of these restriction profiles. In ad-

dition, species with several subtypes, such as *M. gordonae, M. kansasii, M. celatum* and *M. fortuitum* were distinguished.

In the paper of Kim *et al*. (1999), the *rpo*B gene was sequenced in 44 species of mycobacteria. Slowly and fast growing mycobacterial species were differentiated after comparison of the 306 bp nucleotide sequence. The pathogenic *M. kansasii* was easily distinguished from non-pathogenic *M. gastri*, which is not, for example, possible by sequencing of 16S rDNA. About 40 point mutations, deletions and insertions were discovered by sequencing *rpoB*. Point mutations occurred most frequently in the codon, which encodes Ser (531) and His (526). The point mutation Ser (531) to Leu dominated in approximately 70% of Rifampicin-resistant clinical isolates of *M. tuberculosis*.

2.4. Restriction profile analysis of the gene for heat shock protein *hsp* 65

Another possible alternative to commercial methods differentiate the genus *Mycobacterium* is the amplification and subsequent restriction analysis of the gene which encodes the heat shock protein *hsp*65, 65 kDa in size (Plikaytis *et al*., 1992; Telenti *et al*., 1993).

Stress proteins are an important component of the surface antigens of certain pathogens. *Hsp*65 contains both species-specific epitopes and epitopes which regularly occur in different species of mycobacteria. The natural occurrence of conserved sequences of this gene allows the differentiation of mycobacteria through the restriction digestion of PCR products obtained with 'universal' primers Tb11 (5′-ACC AAC GAT GGT GTG TCC AT-3′) and Tb12 (5′-CTT GTC GAA CCG CAT ACC CT-3′) (Telenti *et al*., 1993). A restriction map of the resulting PCR fragment, 441 bp in size, was constructed using the REs *Hae* III and *BstE* II and GCG software (Wisconsin Package, Version 9.1, Genetics Computer Group, USA). In total, 33 different species of mycobacteria were identified by this method (da Silva Rocha *et al*., 1999). On the basis of the two-step digestion (*Hae* III and *BstE* II) of reference strains, Telenti compiled a differentiation algorithm of mycobacteria at the level of species (Telenti *et al*., 1993).

The disadvantage of this method is its inability to differentiate members of the *MTC*. On the other hand, this method distinguishes individual subspecies of the *M. fortuitum* complex. It also separates *MAC* into *M. a. avium* and *M. intracellulare* species, as does the Accu-Probe system. A great benefit is the ability to distinguish between *M. kansasii* and *M. gastri* (Kirschner and Böttger, 1998). However, identification of a series of mycobacteria is complicated because their PCR-REA profiles do not fall into the algorithm compiled on the basis of the PCR-REA patterns of reference strains (da Silva Rocha *et al*., 1999). Recently, new type strains were added to the list of analysed species (Ergin *et al*., 2000; Brunello *et al*., 2001).

2.5. Amplification of the *dna*J gene

The gene for *dna*J encodes a cold-shock protein of 389 amino acids (MW = 41.2 kDa) (Nagai *et al*., 1990). It has been sequenced in 19 mycobacterial species (Takewaki *et al*., 1994). In accordance with Runyon's classic typing (Runyon, 1959), which is based on the pigmentation of colonies and growth speed, phylogenetic relations by sequence comparisons of *dna*J were found inside the three main groups: I, II and III. However, the species *M. simiae*, which is phylogenetically closer to Group III than to Group I is an exception. Fast growing types of mycobacteria, e.g. *M. fortuitum* and *M. chelonae*, did not form a coherent group that is designated by Runyon as group IV. It has been concluded from these results (Takewaki *et al*., 1994) that differentiation on the basis of the sequence of gene *dna*J relates more to the pigmentation of bacterial colonies than to the growth speed of individual types. The same paper proposed a system of digestion of the gene for *dna*J using REs, which enable the differentiation of the majority of mycobacterial types (Takewaki *et al*., 1994).

In the paper of Uematsu *et al*. (1993), a 196 bp fragment of *dna*J gene was used to differentiate of 14 mycobacterial species. Nested PCR was used to obtain the amplicon, and differentiation between species was carried out using PCR with selected REs. A similar strategy has been used in another study to differentiate *M. tuberculosis* from another 11 different species of mycobacteria (Inyaku *et al*., 1993).

In the laboratory in Brno, we use the method of Nagai *et al*. (1990) to differentiate between most species of mycobacteria. We amplify a specific region of *dna*J gene 230 bp in size using the primers YNP9 (5′-GGG TGA CGC GGC ATG GCC CA-3′) and YNP10 (5′-CGG GTT TCG TCG TAC TCC TT-3′). This is a favourable method for the routine differentiation of most mycobacterial species because this region is specific to all species of mycobacteria so far tested.

Based on the sequence of *dna*J, we discovered a method to directly identify a member of the *MAC* down to the subspecies level. PCR with *dna*J specific primers resulted in a non-specific band of 533 bp. This band corresponds to the presence of insertion sequence IS*901* (confirmed by cloning and sequencing). IS*901* is specific for *M. a. avium* (serotypes 1–3), virulent for birds (Pavlík *et al*., 2000b). Presence of the non-specific 533 bp amplicon in addition to the specific 230 bp fragment enables the positive identification of *M. a. avium* with a probability of 65.9%. Absence of the non-specific band indicates that the bacterial strain is, with 99.4% proba-

bility (169 out of 170 samples), IS*901* negative. The non-specific amplicon arises as a result of the primer YNP-9 binding to partly homologous sequences in IS*901* (5′-CCG TAC CGG GT-3′, position 838 bp – 848 bp and 5′-GCC CA-3′, position 1 371 bp – 1 367 bp, Accession No. X59272).

2.6. Using gene sequences (*kat*G, *pnc*A, *oxy*R, *aph*C) for the identification of mycobacteria

From what is known about the molecular-genetic basis of sensitivity of mycobacteria to antituberculotic drugs and other antibacterial medicaments, it is possible to use polymorphism in the corresponding genes to identify and differentiate between mycobacteria. Isoniazid (INH), Pyrazinamide (PZA), Streptomycin (STM), Rifampicin (RFP) and Etambutol (EMB) belong to the basic line of antituberculotic drugs. This chapter will therefore describe genes of mycobacteria which influence the function of some of the chemotherapeutics mentioned above and show variability within the genus *Mycobacterium*.

GENE *kat*G

Pathogenic mycobacteria can survive as intracellular parasites owing to the production of enzymes which degrade the oxygen radicals that form part of the defence mechanism of the parasitised host (Miller *et al*., 1997). Mycobacteria produce catalases and peroxidases which degrade H_2O_2 (Knox, 1956). *MAC* species produce the enzymes KatE, KatG and AhpC. In contrast, only KatG is found in *MTC* species. Protein KatG is a heat-unstable, H_2O_2 inducible enzyme with catalase as well as peroxidase activity. It functions in the transformation of the "prodrug" INH into an active form. Polymorphism in the surrounding regions of the gene *kat*G was used for RFLP analysis of sensitive or resistant *M. tuberculosis* strains by Zhang *et al*. (1993). The sequence of a 75 bp repeat surrounding MPTR, a 10 bp polymorphic tandem repeat, was used as a probe. Since the number of copies of the 75 bp repeat is different in various strains, it is possible to type between them relatively easily using the RFLP method.

In an other study (Brow *et al*., 1996), a 620 bp segment of *kat*G was used for *MTC* differentiation. This segment was denatured and the renaturated single-stranded DNA was digested by the RE *Cleavase* I. This RE digests only a specific single-stranded DNA conformation formed on the basis of its nucleotide sequence. Thus, it is possible to use such digestion to identify point mutations by "structure fingerprints". Structure fingerprints were originally used for studying mutations leading to INH-resistance of *M. tuberculosis* isolates (Brow *et al*., 1996).

GENES *pnc*A *AND oxy*R

PZA is an inactive form of drug which is converted to an active form by the enzyme pyrazinamidase (Pzase). Pzase is encoded by the gene *pnc*A**.** The loss of Pzase activity, which is caused by deletion or substitution, is connected to PZA-resistance (Cheng *et al*., 2000). Together with another gene *oxy*R, which encodes a protein that protects against the oxidative stress response of the host's macrophages, these genes are used for the fast identification of *MTC* strains. The polymorphism of *pnc*A and *oxy*R genes are detected using the PCR-REA method. It is possible to differentiate *M. tuberculosis* from *M. bovis* by using the RE *Alu* I (Yan *et al*., 1998). Through direct sequencing of the 410 bp region of gene *oxy*R in 105 strains of *MTC,* 29 strains were identified as *M. bovis* on the basis of substitution in position 285 of the nucleotide which creates a unique restriction site for the RE *Alu* I. Therefore, it is also possible to differentiate the *M. bovis* species from other *MTC* species using the PCR-RFLP method with the RE *Alu* I.

GENE *ahp*C

Sequence analysis of alleles *rpo*B and *ahp*C was used to study multi-drug-resistant *M. tuberculosis* (MDR-*MTB*) isolates in Scotland between 1990–1997. Gene *ahp*C encodes a detoxifying enzyme which participates in protection from oxidative metabolites. One MDR-*MTB* strain out of these 715 Scottish strains had a synonymous substitution (ATT-ATC) in codon 6 and a very similar IS*6110* RFLP profile to MDR-*MTB* belonging to a group of strains "W", originating from Asia. On the basis of anamnestic data, it was found that this isolate indeed originated from a patient of Asian origin (Fang *et al*., 1999b).

2.7. Analysis of internal transcribed spacer (ITS) 16S-23S rDNA

The rRNA (*rrn)* genetic locus is very interesting from an evolutionary point of view. It is present in both prokaryotes and eukaryotes. In prokaryotes, the *rrn* locus contains genes for all three rRNA types: 16S, 23S and 5S. Genes which encode the relevant rRNA in bacteria are found in 1 to 11 copies (Gurtler and Stanisich, 1996). On the chromosome, genes for rRNA are arranged in groups. Each group of genes for rRNA is transcribed as an rRNA-transcription unit.

Genes for individual types of rRNA are separated by spacers, which demonstrate a high degree of sequence and length variability at the level of genus as well as species. This diversity is caused by variations in the number and types of rRNA sequences which are found inside the spacers. On the basis of substituting genes for tRNA, rRNA-transcription units are marked as: *rrn*A, *rrn*B,

*rrn*C, *rrn*D, *rrn*E a *rrn*F (Gurtler and Stanisich, 1996). The spacer region located between the genes for 16S and 23S rRNA is extremely variable even in terms of closely related taxonomic groups, which is a result of frequent insertions and deletions in this region of the genome (Gurtler and Stanisich, 1996).

The polymorphism in the length and sequences of spacers in the *rrn* locus is used to differentiate various types of prokaryotes (Barry *et al*., 1990). Jensen *et al*. (1993) amplified the spacer 16S-23S in 300 bacterial species which belonged to 8 genera and 28 species or serotypes. For the purposes of amplification they used primer G1 (5′-GAA GTC GTA ACA AGG-3′), whose sequence had been derived from the highly conserved region 16S rDNA immediately adjacent to the spacer 16S-23S, and primer L1 (5′-CAA GGC ATC CAC CGT-3′), which had been derived from the most conserved part of the 23S rDNA sequence, located just behind the spacer. All 28 tested species displayed characteristic amplification profiles.

By sequence analysis of the internal transcribed spacer (ITS) 16S-23S rDNA it is possible to separate certain species of mycobacteria into specific intraspecies taxons called "sequevars", e.g. five sequevars were identified in the *MAC* species: MavA to MavE, which differ in one or two nucleotides (Novi *et al*., 2000). ITS sequencing supplements information acquired by sequencing 16S rDNA, which can be used to differentiate closely related species. A high sequence variability in the ITS of slowly growing mycobacteria has been noted (Roth *et al*., 1998). On the basis of this high degree of ITS variability, closely related species such as *M. gastri* and *M. kansasii* were identified, which cannot be differentiated using sequence analysis of the gene for 16S rDNA. However, ITS analysis failed in the discrimination between *M. marinum* and *M. ulcerans*, which share a high degree of genome relatedness, also demonstrated by 16S rDNA analysis. Moreover, the same ITS sequences were discovered in members of the *MTC*, which includes phylogenetically distant bacterial species.

Roth *et al*. (1998) analysed the ITS sequence of 60 strains of the genus *Mycobacterium*, which included 13 species. Using PCR amplification with the primers Ec16S.1390b (5′-TTG TAC ACA CCG CCC GTC A-3′) and Mb23S.44n (5′-TCT CGA TGC CAA GGC ATC CAC C-3′), which had been derived from the adjacent regions of the genes 16S rDNA and 23S rDNA, they detected a PCR product 480 bp in size in all 60 strains. Using sequence analysis they found that the spacer sizes of slowly growing species of mycobacteria are in the range from 235 nucleotides in *M. xenopi* to 285 in *M. gastri*. They were on average 75 nucleotides shorter than in fast growing species of mycobacteria. On the basis of this difference in ITS length it is possible to distinguish visibly slowly growing and fast growing strains. Sectors with a high degree of sequence variability are dispensed over the whole spacer sequence. A consequence of this significant variability was the discovery of intraspecies sequence polymorphism in 4 of the 11 species, in *M. gastri* designated as sequevars MgaA and MgaB, MAC (MavA to MavE), *M. simiae* (MsiA to MsiD) and *M. xenopi* (MxeA to MxeC).

Furthermore, a PCR-REA method on the basis of ITS was used to distinguish *MTC* from *MAC* (Sansila *et al*., 1998). In a first PCR step, a 380 bp product was amplified using primers 16SC (5′-TCG AAG GTG GGA TCG GC-3′) and 23SG (5′-GCG CCC TTA GAC ACT TAC-3′), which had been derived from adjacent sequences of rDNA. Afterwards, this product was digested with RE *Hae* III, *Msp* I or *Bst*X I (Sansila *et al*., 1998). After the digestion with RE *Hae* III, unique PCR-REA profiles were obtained for different *MAC* species. PCR-REA constituents of *M. intracellulare* were similar to patterns of *M. scrofulaceum*. A significant disadvantage of this method was the inability to differentiate individual *MTC* subtypes.

A subsequent paper (Roth *et al*., 2000) describes a new diagnostic algorithm for differentiation of mycobacteria by PCR-REA. After amplification with primer Sp1 (5′-ACC TCC TTT CTA AGG AGC ACC-3′), which had been derived from the beginning of the *M. tuberculosis* spacer sequence (Accession No. L15623) and primer Sp2 derived from position 210 bp to 190 bp (5′-GAT GCT CGC AAC CAC TAT CCA-3′), amplicons of different lengths were obtained. In slowly growing mycobacteria, the size of amplicons are between 200 bp and 330 bp, in fast growing species the fragments were always longer than 250 bp. Using the REs *Hae* III and *Cfo* I, it was possible to differentiate slowly and fast growing mycobacteria. Fast growing species displayed PCR-RFLP fragments in the range 33 bp to 230 bp larger than slowly growing species (which produced products in the range 33 bp to 175 bp) after digestion by RE *Hae* III. Digestion by RE *Cfo* I resulted in longer fragments from fast growing species, which contained a specific restriction site. A disadvantage was that a great number of fast growing species of mycobacteria did not have a restriction site for RE *Cfo* I at all. In contrast, the amplified region of DNA of all species of slowly growing bacteria were able to be digested by this RE. The amplified region of DNA from slowly growing bacteria carried a specific restriction site for RE *Cfo* I and formed shorter fragments divided into groups A to D. For the final determination of species belonging to the fast growing mycobacteria, either the RE *Taq* I was used, namely for the differentiation of *M. abscessus* from *M. chelonae* I, II and III, or RE *Ava* II for the differentiation of *M. porcinum* from *M. farcinogenes*. REs *Dde* I, *Taq* I or *Ava* II were used for precise differentiation of slowly growing species of mycobacteria. It was necessary to use yet another step with certain species of this group of mycobacteria, namely digestion by REs *Msp* I (*M. simiae*) and *Hinf* I (*M. kansasii*). The algorithm for slowly growing species of mycobacteria

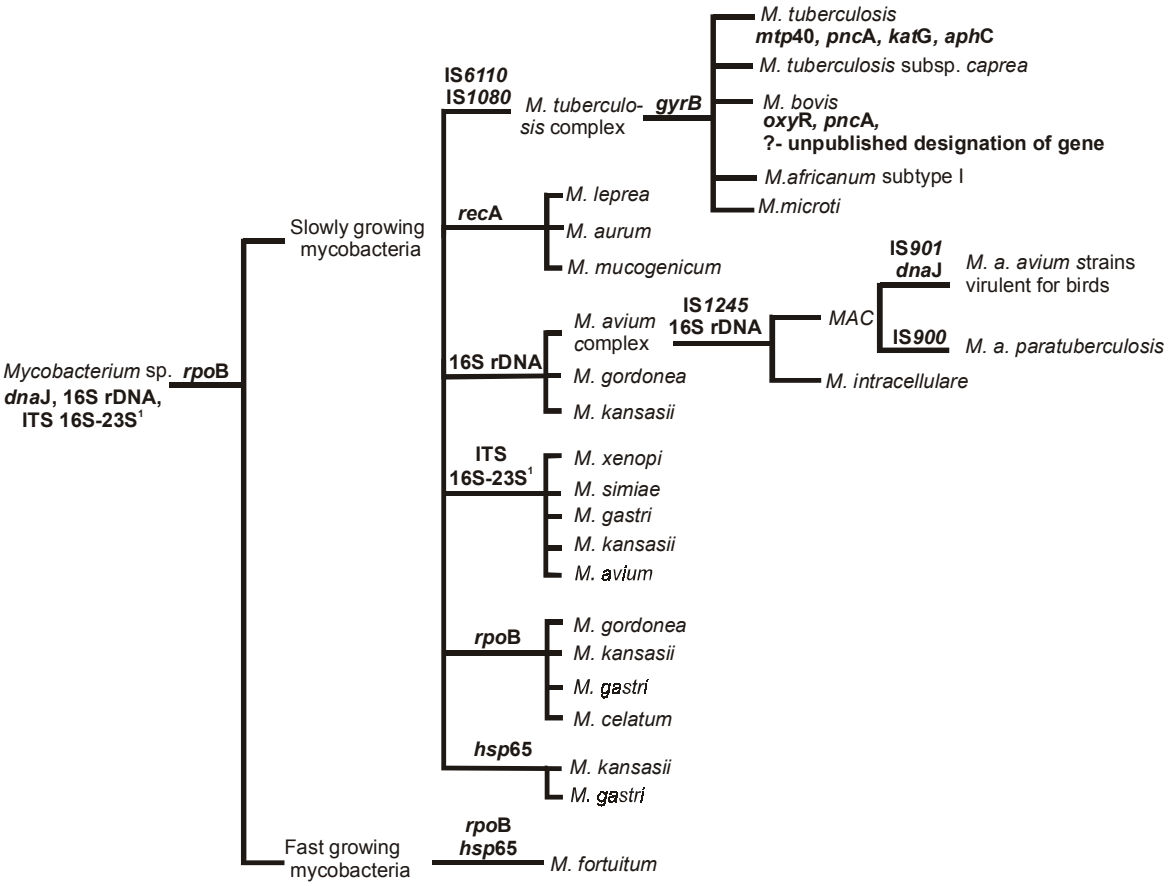

Figure 2. Diagnostic algorithm for the identification and differentiation of medically important mycobacterial species (Table 1)

[1]internal transcribed space 16S-23S rDNA, *MAC* – *M. avium* complex

thus became very complex and difficult to interpret. Problems also arise with the fast growing species of mycobacteria with the interpretation of so far unclearly defined species (Roth *et al*., 2000).

3. METHODS FOR DIFFERENTIATION AND IDENTIFICATION OF THE *M. AVIUM* SUBTYPES

Currently used molecular techniques, especially the RFLP method, provide a new way of looking at strains of individual *MAC* species (McFadden *et al*., 1987). Insertion sequences, which vary in the number of copies and their locations on the genome, are used as probes to type species (van Soolingen *et al*., 1998a). IS are characterised by a very low degree of mobility and limited polymorphism. Thus they have become an appropriate tool in the study of epidemiology of mycobacterial infections (Pavlík *et al*., 1999a; Dvorská *et al*., 2002).

The discovery of insertion sequences in mycobacterial genomes, e.g. IS*900* in *M. a. paratuberculosis* (Green *et al*., 1989), IS*901* (Kunze *et al*., 1991), IS*1245* (Guerrero *et al*., 1995), IS*1311* (Roiz *et al*., 1995) in the *MAC* strains was a major breakthrough in the study of mycobacterial infections. A summary about mycobacterial ISs is given in our previous study (Dvorská *et al*., 1998). Up-to-date information about the number of mycobacterial insertion sequences is available in the international database of IS at: http://pc4.sisc.ucl.ac.be/is.html

3.1. Insertion sequence IS*900* specific for *M. a. paratuberculosis*

The occurrence of IS*900* is limited only to the *M. a. paratuberculosis* genome, where it is found in 15 to 20 copies (Green *et al*., 1989; Pavlík *et al*., 1995, 1999c; Bull *et al*., 2000). IS*900* is 1451 bp in size and has one open reading frame (ORF) which encodes the protein p43. From an epidemiological point of view, IS*900* is a highly stable marker used for precise identification of *M. a. paratuberculosis* by the PCR method. Various locations and numbers of IS*900* on the genome are then

used for the typing of individual strains using the RFLP method (Kunze *et al*., 1991; Pavlík *et al*., 1999c).

In the laboratory in Brno we drew up a standardisation scheme for typing individual strains of *M. a. paratuberculosis* with the RFLP method using parallel digestion by two REs *Pst* I and *BstE* II (Pavlík *et al*., 1999c). For the description of a wide spectrum of different RFLP types we applied computer analysis of DNA fingerprints using the Gel Compar software (Applied Maths, Kortrijk, Belgium). The obtained results and identification scheme are available at:
http://www.vri.cz:/wwwrflptext.htm.

Thirteen *Pst* I RFLP types designated A to M were identified in 1 008 strains of *M. a. paratuberculosis* using this system. Twenty RFLP types were detected by parallel digestion of chromosomal DNA by the RE *BstE* II. Eighteen fingerprints: C1 to C3, C5, C7 to C20 were included in a group of RFLP types designated with the letter C (cattle). One RFLP type S1 was detected in a group of RFLP types designated S (sheep) and one type I1 was also detected in a group of RFLP type I (intermediate). Previously described RFLP types C4, C6 and S2 (Collins *et al*., 1990) and RFLP types S3 and I2 (deLisle *et al*., 1993) were included into our typing system after scanning them from published figures. Using a combination of results obtained after the parallel digestion by *Pst* I and *BstE* II, a total of 28 different RFLP types were differentiated (Pavlík *et al*., 1999c). This method was used in several epidemiological studies (Pavlík *et al*., 1994, 1996, 1999b,d, 2000a; Greig *et al*., 1999; Whitlock *et al*., 1999).

3.2. Identification of avian tuberculosis caused by IS*901*+ strains

The insertion sequence IS*901* was discovered by Kunze *et al*. (1991). It is 1472 bp in size, and contains one ORF for transponase, a protein of 401 amino acids. The stability of IS*901* in strains isolated primarily from clinical material from birds, domestic animals and from the environment is used for the rapid identification of IS*901*+ strains using the PCR method. IS*901* occurs on the genome of virulent *MAC* strains in 2 to 13 copies (Kunze *et al*., 1991; Ritacco *et al*., 1998), which we also confirmed in 172 strains examined (Dvorská *et al*., 2002).

In in the laboratory in Brno, we explored the relationship between the presence of IS*901* (PCR) and the virulence of these strains for birds in a biological experiment. A total 165 out of 738 (22.4%) strains caused generalised tuberculosis, 164 (99.4%) of them contained IS*901*. The remaining 573 (77.6%) strains were non-virulent; however, IS*901* was present in 24 (4.2%) strains. As the majority of these strains were from collections, they might have lost virulence during several decades of storage. IS*901* was demonstrated in all virulent field strains, which enabled the replacement of biological experiments by the PCR method.

3.3. Identification of avian mycobacterioses caused by IS*1245*+ strains

The presence of IS*1245* has often been used to identify *MAC* strains (Guerrero *et al*., 1995; Ritacco *et al*., 1998). IS*1245* is limited to the species *M. a. avium*, *M. a. paratuberculosis* and *M. a. silvaticum* strains (so-called "wood pigeon" strains). Strains of *M. intracellulare* entirely lack this genomic element (Guerrero *et al*., 1995). IS*1245* has been found in more than 20 copies in isolates from people and pigs. In contrast, *MAC* strains virulent for poultry (containing IS*901*), contained only three copies of this element, so-called "bird type" (Ritacco *et al*., 1998). Furthermore, it was observed that the number of IS*1245* copies on the genome relates to the serotype of the given strain. The "bird type" (three IS*1245* bands) contains strains of the serotypes 1 to 3. Strains of serotypes 4 to 6, 8 to 11 and 21 formed polymorphous IS*1245* RFLP profiles of 6 and 20 bands. Strains of serotypes 7, 12 to 20, 22 to 28 did not contain element IS*1245* at all (Ritacco *et al*., 1998).

3.4. Differentiation of *M. a. avium* and *M. a. paratuberculosis* using other methods

Eriks *et al*. (1996) developed a method to differentiate *M. a. avium* and *M. a. paratuberculosis* on the basis of PCR-REA. They digested a 960 bp amplicon of the gene *hsp*65 using the RE *Pst* I. It was possible to differentiate clinical isolates of both subspecies. The method was particularly useful to classify strain 18 of *M. a. avium*, which carried the insertion sequence IS*901* and was incorrectly classified as *M. a. paratuberculosis* (Merkal, 1979; Chiodini, 1993).

4. METHODS OF DIFFERENTIATING THE *M. TUBERCULOSIS* COMPLEX (*MTC*)

4.1. Detection of repeaed sequences of DNA

Members of the *MTC* (*M. tuberculosis, M. africanum, M. bovis, M. bovis* BCG, *M. microti, M. tuberculosis* subsp. *caprae* and *M. canetti*) are genetically closely related taxons. The relatively high degree of intertype DNA homology of *MTC* strains in the 16S rDNA sequence and of the 16S-23S spacer limits the differentiation of these types using restriction analysis (Eisenach *et al*., 1988). Despite this genetic homology in the *MTC*, a relatively high DNA polymorphism has been found in repetitive DNA, which are ISs (Dvorská *et al*., 1998). Up till now,

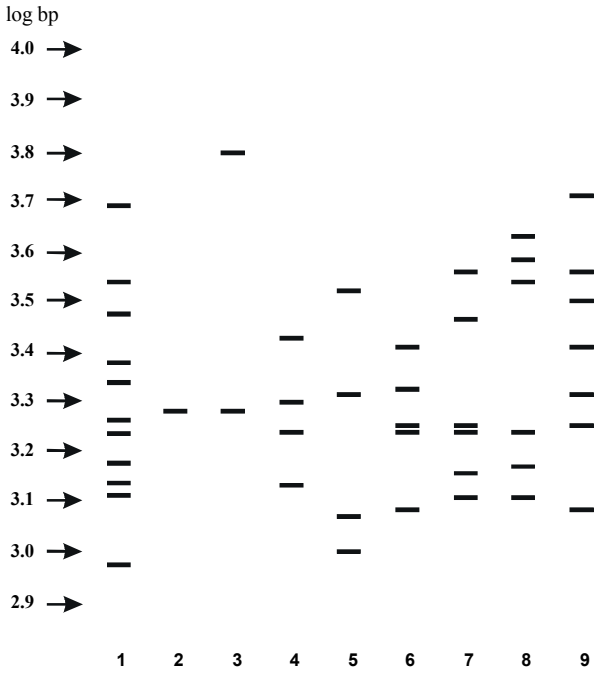

Figure 3. IS*6110* RFLP pattern of *M. tuberculosis* (line 1), *M. bovis* BCG (line 2), *M. bovis* (lines 3 to 5) and *M. tuberculosis* subsp. *caprae* (lines 6 to 9)

the following IS have been identified in *MTC* strains: IS*6110* (Thierry *et al*., 1990), IS*1081* (Collins and Stephens, 1991), IS*1547* (Fang *et al*., 1999a) and an IS-like element (Mariani *et al*., 1993).

For *MTC,* IS*6110* RFLP has become a widely used typing method for epidemiological studies. An example of typical pattern is given in Figure 3. In *M. tuberculosis* isolates (line 1) the large number of IS*6110* copies on the genome permits an excellent use of this element for strain comparison in outbreak tracing. *M. bovis* strains often contain less than five IS*6110* copies (Figure 3: BCG with one copy and three examples of different cattle outbreak strains from Germany). Therefore, the use of IS*6110* RFLP for epidemiological studies in *M. bovis* is more limited and was more successful by combination with spoligotyping (Chapter 4.2.). As also *M. tuberculosis* strains with a low number of IS*6110* copies exist, the RFLP pattern can only be used for differentiation between the species with a probability of error. In *M. tuberculosis* subsp. *caprae* (*M. t. caprae*) the usual copy number of IS*6110* appears to be higher than in *M. bovis* (last 4 lines). IS*6110* is suitable to subtype spoligopattern which are more conserved in *M. t. caprae* (unpublished observation).

In contrast to IS*6110*, the use of the remaining IS elements is limited by the small number of copies on the genome and the low degree of polymorphism (van Soolingen *et al*., 1998b). However, six other types of short repetitive DNA with a varying degree of genetic diversity and potential usefulness were identified: DR (Thierry *et al*., 1990), PGRS (Ross *et al*., 1992), GTG and MPTR (Hermans *et al*., 1992), ETR and VNTR (Frothingham and Meeker-O'Connell, 1998). Kremer *et al*. (1999) compared 12 current molecular methods to distinguish *MTC* subtypes based on RFLP, DNA hybridisation, PCR and combinations of these methods. In *MTC*, the intention of some methods is differentiation and typing in the sense of definition, as mentioned for IS*6110* already and as will be shown for spoligotyping later. By studying 60 *MTC* strains, Kremer *et al*. (1999) showed that the IS*6110* RFLP and mixed-linker PCR have the greatest discriminatory capability in strains with a high number of IS*6110* copies. The mixed-linker PCR method is based on a primer specific to the IS*6110* and a primer which is complementary to the linker attached by ligation to restriction fragments of genomic DNA. Strains with between 5 and 7 IS*6110* copies (*M. bovis* and *M. tuberculosis* strains isolated in Asia) may be best typed using VNTR, PGRS-RFLP, DR-RFLP or by spoligotyping. For strains with one or two IS*6110* copies, the most effective methods are PGRS-RFLP and DR-RFLP. Since two strains of *MTC* were identified which did not carry at all IS*6110*, the spoligotyping method was applied to differentiate them.

In the paper of O'Brien *et al*. (2000), 60 strains of *M. bovis* isolated from 1993 to 1998 from cattle, pigs, deer and badger contained a single copy of IS*6110*. A combination of PGRS-RFLP, DR-RFLP and spoligotyping was used to type them. For DNA fingerprinting, a plasmid containing a 4 kb insert from restriction digestion of *M. bovis* DNA with the RE *Alu* I was used as a probe. By comparing the sequence of the insert with the whole genome of *M. tuberculosis* strains H37Rv using the BLAST database, it was found that it is 90% homologous with the PPE gene (gene Rv0096). This gene consists of a large number of repetitive 69 bp units. The resulting fingerprints were readily distinguishable. 18 types were distinguished using a DNA probe on the basis of a PPE gene, 11 types using the PGRS-RFLP method, 10 types using DR-RFLP and 8 types using spoligotyping. Using a combination of the hybridisation results with the gene for PPE and DR-RFLP, 26 types were detected, and finally using a combination of all four methods, 28 types were distinguished. For expediency and adequate typing capabilities, the authors recommend the combination PPE-DR.

4.2. Differentiation and typing of strains of the *MTC* by spoligotyping

Spoligotyping is based on DNA polymorphism at a chromosomal locus, which is characterised by the presence of a high number of conserved direct repeats, designated as the DR region (Thierry *et al*., 1990). The direct repeats are 36 bp in size and are interrupted by DNA spacers of 35 bp to 41 bp. When the DR regions of several

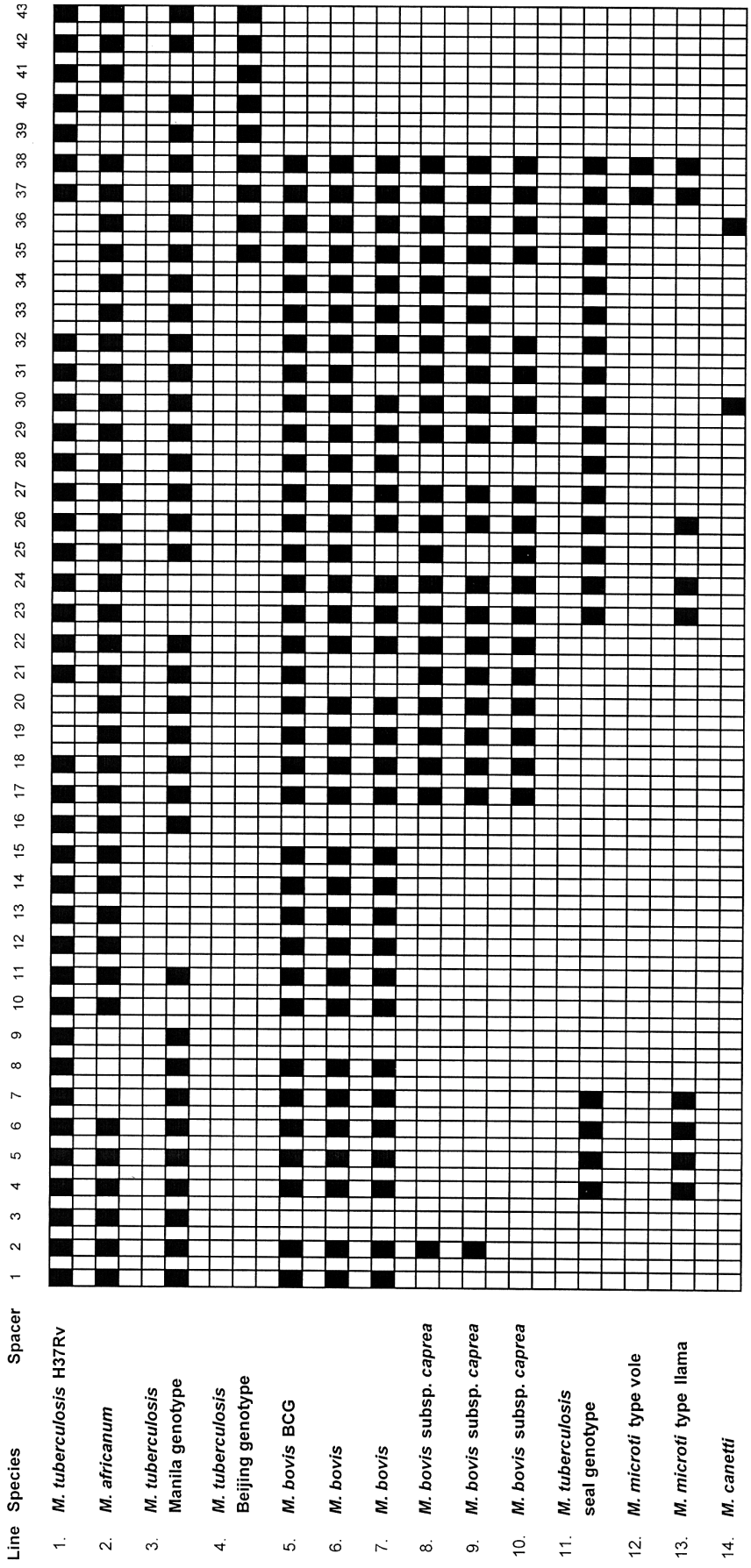

Figure 4. Spoligopatterns typical for *MTC* species and subtypes (van Soolingen *et al.*, 1997, 1998b; Aranaz *et al.*, 1999; Zumarrage *et al.*, 1999; Niemann *et al.*, 2000b; Sola *et al.*, 2000; Viana-Niero *et al.*, 2001)

strains were compared, it was noted that the order of spacers is nearly the same in all strains, but that there may be deletions or insertions. The presence or absence of 43 individual spacers may be detected using the spoligotyping method.

For the purposes of spoligotyping, which is in fact a reverse hybridisation, all 43 oligonucleotides are bound covalently to a nylon membrane in parallel rows. Each of the oligonucleotides immobilised in this way represents the unique sequence of a spacer which is a component of the DR region in *MTC*. In total, 37 oligonucleotides were derived from the sequences of spacers present in the *M. tuberculosis* H37Rv and the remaining six sequences of oligonucleotides were derived from the spacers of *M. bovis* BCG. These loaded membranes are commercially available.

To perform the method in the laboratory, initially the DR region including the various spacers has to be amplified by PCR. The primers are derived from the DR sequence. A biotinilated reverse primer is used to label the PCR products which after heat-denaturation are hybridised to the oligonucleotides on the membrane and detected by chemoluminescence. The presence of a spacer appears on the autoradiographic film as a signal in the shape of a black square. Individual strains are characterised by a grouping of squares, which represent the existing spacers.

Spoligotyping is an excellent method for differentiation of *MTC* subtypes based on the presence and/or absence of certain combinations of spacers. The types which can be differentiated are given in Figure 4 which summarises own results and the literature (van Soolingen *et al*., 1997, 1998b; Zumarraga *et al*., 1999; Aranaz *et al*., 1999; Niemann *et al*., 2000a,b; Sola *et al*., 2000; Viana-Niero *et al*., 2001). The *M. tuberculosis* subtypes (strain H37Rv is shown in line 1) are particularly characterised by the presence of spacers 39–43. Individual differences in *M. tuberculosis* strains occur in the presence/absence of spacers 1–38. If the spacers 7, 8, 9 and 39 are missing, an isolate falls into subtype *M. africanum* (line 2); *M. tuberculosis* Manila genotype (line 3) is characterised by the missing of spacer 41 and more than one of the lower spacers. *M. tuberculosis* Beijing genotype (line 4) is characterised by the presence of spacers 35 to 43 only.

All following species in Figure 4 (lines 5 to 14) are characterised by the absence of spacers 3, 9, 16 and 39–43, which is typical for *M. bovis*. Included in the Figure 4 are *M. bovis* BCG (line 5) and two isolates from cattle outbreaks in Germany (lines 6 and 7). The differences in the spoligopattern (spacers 21, 25 and 31) allow a strain identical description. This illustrates that spoligotyping is not only a technique for differentiation but also for typing of strains for epidemiological analyses. This is very useful for the subtypes *M. tuberculosis*, *M. bovis* and *M. t. caprae*. Subspecies *M. t. caprae*, represented by 3 different isolates from cattle (lines 8 to 10), is particularly characterised by the absence of spacers 3 to 16. The absence of the spacers 3, 9, 16 and 39–43 illustrates the close relationship to *M. bovis.*

In the last decades, 4 *MTC* subtypes have been identified which differ significantly from typical *MTC* growth and biochemistry or have been found to be linked to individual host species. Moreover, they are characterised by unique spoligopatterns. *M. tuberculosis* seal genotype (line 11) was found in different species of seals in South America (Zamarraga *et al*., 1999) and is characterised by the absence of spacers 8 to 22. *M. microti* (line 12), originally isolated from tuberculosis in wild vole, has been isolated also from other animal species and human tuberculosis cases (van Soolingen *et al*., 1998b). It is existing in two types*: M. microti* presence of spacer 37 and 38 only and the llama-type 37–38 plus 26, 23–24 and 4–7. The species *M. canetti*, which has been isolated in only a few occasions so far (van Soolingen *et al*., 1997) is characterised by the presence of only two spacers: 30 and 36.

As spoligotyping is a PCR driven technique, only small amounts of DNA are required for analysis. Therefore, spoligotyping is particularly suitable for the analysis of slowly growing mycobacteria. It also permits the comparison of strains which are not culturable after prolonged storage. This can be of irreplaceable importance, as in the case of relapses, when it is necessary to compare new strains from patients whose earlier isolates can no longer be cultivated.

We used spoligotyping to examine the DNA of 24 dead isolates of *M. bovis* collected from 1965 to 1999 (Pavlík *et al*., 2001). 22 isolates from the Czech Republic, and one isolate from Slovakia and a Neotype *M. bovis* strain ATCC 19210 were examined. The spoligopatterns were evaluated using the software Gel Compare (Applied Maths, Version 4.1, Kortrijk, Belgium). Seven spoligotypes with the working designates S1 to S7 were found (Figure 5). These were compared with the spoligotypes of 3 176 isolates from the RIVM (Netherlands Institute of Public Health and the Environment, Bilthoven, The Netherlands) database. The Neotype *M. bovis* strain stored in the Czech collection CNCTC (My310/82) since 1974 was of the identical spoligotype S4 with an original Neotype strain from the USA. Isolates from the South American capybara (*Hydrochoerus hydrochaeris*), imported from Germany in 1989, from cattle (isolated in 1966, 1991 and 1994) and from three children with post-vaccination complications from the BCG vaccine were of the most common spoligotype S1. Four unique spoligotypes S2, S3, S5 and S6 were identified in isolates from the Czech Republic from cattle (1965 and 1974), from a farm-reared red deer (*Cervus elaphus*) and from Slovakia from cattle (1992). Czech isolates from a wild red deer (1991), from cattle (1966, 1991, 1995) and from an eighty-year-old man (1999) were described to the sporadically occurring spoligotype S7. Later it was found

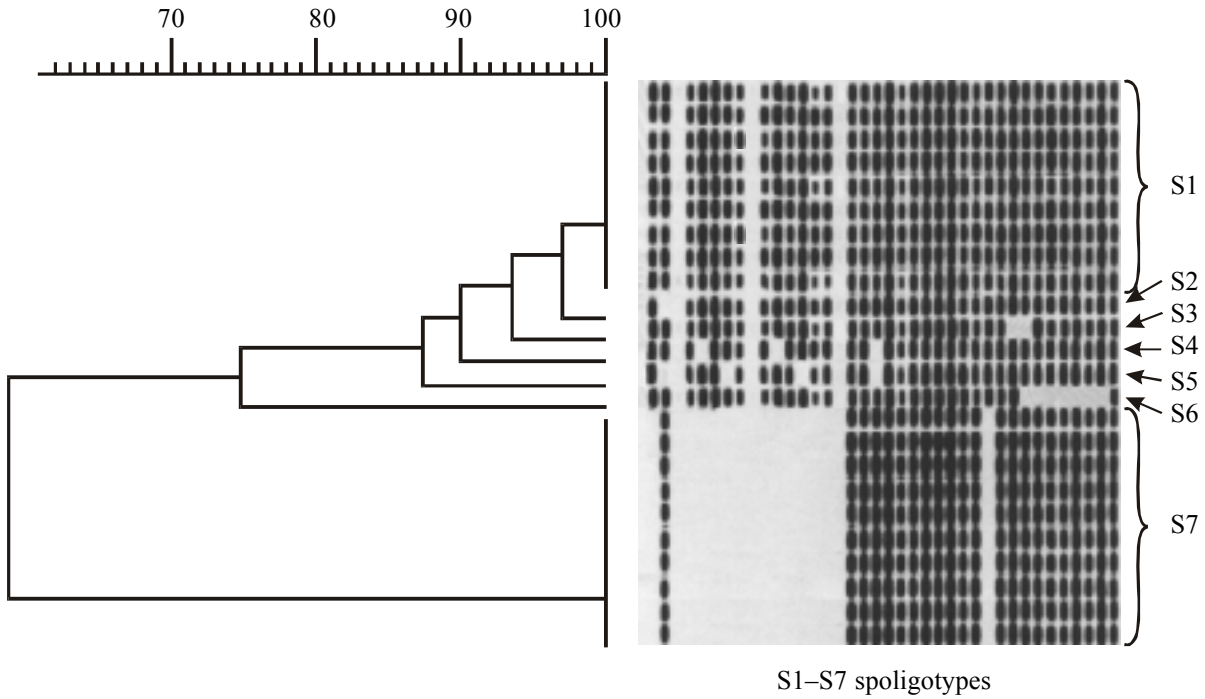

Figure 5. Dendrogram of spoligotypes of strains from the years 1965–1999 (Pavlík *et al*., 2001)

that the S7 profile corresponds to the spoligotype *M. t. caprae* (Aranaz *et al*., 1999). In the Czech Republic, isolates of both unique spoligotypes and the most common spoligotype in the RIVM database were found in infected cattle focuses in the years 1965 to 1995 (Pavlík *et al*., 2001).

4.3. Differentiation of *M. tuberculosis* and *M. bovis* by the detection of specific genes (*mtp*40) and gene variation (*oxyr*R and *pnc*A)

Strains of the species *M. tuberculosis* can be identified by the detection of the species-specific fragment *mtp*40 using the PCR method with the primers PT1 (5′-CAA CGC GCC GTC GGT GG-3′) and PT2 (5′-CCC CCC ACG GCA CCG C-3′). The resulting 392 bp fragment corresponds to a surface antigen which is only present in the genome of *M. tuberculosis*, and is not found in genomes of other species of the *MTC*. This property was used for the development of a specific, sensitive and rapid diagnostic test by which infection caused by strains of *M. tuberculosis* could be distinguished from infection by strains of *M. bovis* (del Portillo *et al*., 1991; Herrera and Segovia, 1996). Using the PCR method of the gene *mtp*40, it is also possible to detect strains of *M. tuberculosis* which do not carry any copy of IS*6110* in its genome (Herrera and Segovia, 1996).

In samples with a negative amplification it is possible, through a subsequent amplification using primers specific to *M. bovis* L1 (5′-CCC GCT GAT GCA AGT GCC-3′) and L2 (5′-CCC GCA CAT CCC AAC ACC-3′) to identify this *MTC* species (Romero *et al*., 1999). It is also possible to distinguish individual *MTC* species through sequence and restriction analyses of the genes *oxy*R, *pnc*A a *rpo*B (Chapter 2.6.).

4.4. Differentiation of *MTC*-subspecies by restriction analysis of DNA sequence *gyr*B

DNA gyrase is a topoisomerase II; a class of ATP-dependent enzymes which are capable of creating negative superhelixes from relaxed forms of covalently closed circle (CCC) plasmid DNAs. Topoisomerases participate in the regulation and progression of many important cellular functions (particularly replication, transcription and recombination). DNA gyrase is composed of two subunits A and B, the encoding genes are *gyr*A and *gyr*B. Both genes are found in a 5 119 bp region of DNA. The proteins GyrB (75 kDa) and GyrA (95 kDa) in mycobacteria demonstrate 45–80% identity to the gyrases of other bacteria (Unniraman and Nagaraja, 1999). Polymorphisms in the sequence of *gyr*B were discovered and subsequently used to differentiate between *MTC* species. Amplification fragments, 1 200 bp in size, obtained from clinical isolates of *M. tuberculosis, M. bovis* (PZA-sensitive and -resistant strains), *M. africanum* subtypes I and II and *M. microti* types vole and Ilama were sequenced (Niemann *et al*., 2000a) or examined using the PCR-

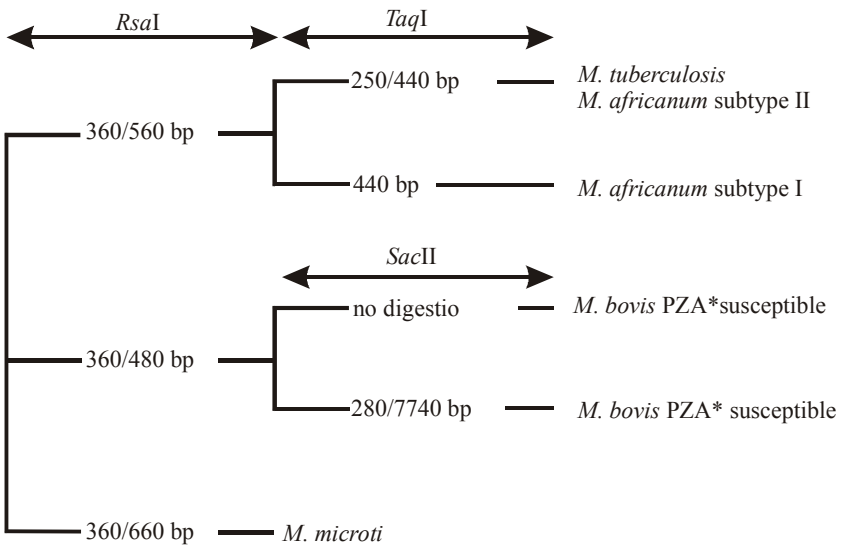

Figure 6. Diagnostic algorithm of PCR-REA patterns for the differentiation of members of *MTC* by *gyr*B (Niemann *et al*., 2000a)

*PZA – Pyrazinamide

REA method (Kasai *et al*., 2000; Niemann *et al*., 2000a). Using sequence analysis, polymorphisms were discovered in five regions of the gene *gyr*B. Strains of *M. bovis* carried a specific substitution in *gyr*B, through which this species may be differentiated from other *MTC* species. Strains of *M. tuberculosis* and *M. africanum* subtype II had the same *gyr*B sequence. The subspecies *M. africanum* subtype I has a unique substitution at position 1 450 in the sequence of *gyr*B. While isolates of *M. microti* type llama and vole could be distinguished from other species, individual strains could not be differentiated (Niemann *et al*., 2000a).

To differentiate *MTC* subspecies on the basis of the *gyr*B sequence, Kasai *et al*. (2000) and Niemann *et al*. (2000a) cut a PCR product of 1 020 bp using the REs *Rsa* I, *Taq* I and *Sac* II. On the basis of the restriction fragments, a diagnostic algorithm was developed for the easy differentiation of *MTC* species (Figure 6). All iso-

Table 1. Possibility of differentiation of genus *Mycobacterium*, *MAC* and *MTC* and species of other mycobacteria on the DNA level (Figure 2)

Differentiation of mycobacterial genus/complex/species	Gene	Method	Reference
Mycobacterium sp.	16S rDNA	PCR-REA multiplex PCR sequencing analysis	Thierry *et al*. (1990) Wilton and Cousins (1992) Kirscher and Böttger (1998)
Mycobacterium sp.	ITS 16S-23S rDNA	PCR-REA	Roth *et al*. (2000)
Mycobacterium sp.	*dna*J	PCR	Nagai *et al*. (1990)
Runyon's Groups Runyon (1959)	*dna*J	sequencing analysis PCR-REA	Takewai *et al*. (1993)
Groups of fast and slowly growing mycobacteria	*rpo*B	sequencing analysis	Kim *et al*. (1999)
M. avium complex	IS*1245*	PCR RFLP	Guerrero *et al.* (1995) Ritacco *et al*. (1998)
M. a. avium	IS*901* 16S rDNA	PCR, RFLP multiplex PCR	Kunze *et al*. (1991) Wilton and Cousins (1992)
M. intracellulare	16S rDNA ITS 16S-23S rDNA	multiplex PCR PCR-REA sequencing analysis	Wilton and Cousins (1992) Vaneechoutte *et al*. (1993) Sansila *et al*. (1998)
M. a. paratuberculosis	16S rDNA IS*900*	multiplex PCR PCR RFLP	Wilton and Cousins (1992) Green *et al*. (1989) Pavlík *et al*. (1999c)

Table 1 to be continued

Differentiation of mycobacterial genus/complex/species	Gene	Method	Reference
M. tuberculosis complex	DR	PCR, RFLP, spoligotyping	Thierry *et al*. (1990, 1998b) van Soolingen *et al*. (1997) Zumarraga *et al*. (1999) Aranaz *et al*. (1999) Niemann *et al*. (2000b) Sola *et al*. (2000) Viana-Niero *et al*. (2001)
	IS6*110*	PCR	Thierry *et al*. (1990)
		RFLP	Van Soolingen *et al*. (1991)
	IS*1081*	PCR, RFLP	Collins and Stephens (1991)
	PGRS	PCR, RFLP	Ross *et al*. (1992)
	MPTR, GTG	PCR, RFLP	Hermans *et al*. (1992)
	ETR VNTR	PCR, RFLP PCR, RFLP	Frothingham and Meeker–O'Connell (1998)
	*gyr*B	sequencing analysis	Niemann *et al*. (2000a)
		PCR-REA	Kasai *et al*. (2000)
M. bovis	*oxy*R	sequencing analysis	Yan *et al*. (1998)
	*pnc*A	PCR-REA	Yan *et al*. (1998)
	?	PCR	Romero *et al*. (1999)
M. tuberculosis	*mtp*40	PCR	del Portillo *et al*. (1991)
	*pnc*A	PCR-REA	Yan *et al*. (1998)
M. tuberculosis INH-resistant strains	*kat*G	structural fingerprinting RFLP	Zhang *et al*. (1993) Brow *et al*. (1996)
M. tuberculosis MDR strains	*aph*C	sequencing analysis	Fang *et al*. (1999)
M. fortuitum	*hsp*65	PCR-REA	Telenti *et al*. (1993)
	*rpo*B	PCR-REA	Kim *et al*. (1999) Lee *et al*. (2000)
M. celatum	*rpo*B	PCR-REA	Kim *et al*. (1999) Lee *et al*. (2000)
M. gordonae	*rpo*B	PCR-REA	Kim *et al*. (1999) Lee *et al*. (2000)
	16S rDNA	sequencing analysis	Kirscher and Böttger (1998)
M. kansasii	*hsp*65	PCR-REA	Telenti *et al*. (1993)
	16S rDNA	sequencing analysis	Kirscher and Böttger (1998)
	*rpo*B	PCR-REA	Kim *et al*. (1999)
		sequencing analysis	Lee *et al.* (2000)
M. gastri	*hsp*65	PCR-REA	Telenti *et al*. (1993)
	*rpo*B	sequencing analysis	Kim et *al*. (1999)
M. leprae *M. aurum* *M. mucogenicum*	*rec*A	sequencing analysis	Blackwood *et al*. (2000)
Sequevars *M. avium* Sequevars *M. simiae* Sequevars *M. xenopi* Sequevars *M. gastri*	ITS 16S-23S rDNA	sequencing analysis	Roth *et al*. (1998) Novi *et al*. (2000)

? – unpublished designation of gene; *a*. – *avium*; INH – Isoniazid; ITS - Internal Transcribed Spacer 16S-23S rDNA; MDR – multidrug-resistant strains

lates of *M. tuberculosis* displayed typical *Rsa* I-*Taq* I RFLP profiles. Likewise, isolates of PZA-resistant *M. bovis* created unique *Rsa* I-*Sac* II RFLP profiles. Taken together, the PCR-REA with the combination of REs described above permits the easy and rapid differentiation between the species *M. tuberculosis/M. africanum* type II, *M. africanum* type I, *M. microti*, and *M. bovis.* However, it does not differentiate the *M. tuberculosis* from *M. africanum* type II.

4.5. Identification of Rifampicin-resistant *M. tuberculosis* strains by oligonucleotide microchips

In response to the spread of MDR-*MTB*, there is a growing interest in developing methods for its rapid identification. A new approach described by Mikhailovich *et al.* (2001) enables the identification of drug-resistant strains on microchips. These microchips are composed of oligonucleotide probes immobilised on polyacrylamide blocks. Using DNA hybridisation, PCR and also a ligase-chain reaction, analysis of the point mutations within a variable 81 bp long domain of the *rpo* gene was carried out on a total of 42 oligonucleotides. This microchip approach contains precisely the mutations in this domain which are responsible for 95% of the cases of RFP-resistance. Advantages include the exceptional speed of analysis (approximately 1.5 hrs) and the sensitivity. For example, in a mixture of bacterial cells, as little as 1% of MDR-*MTB* could be detected by the chip.

5. CONCLUSION

A limited number of biochemical tests are used to differentiate and identify mycobacteria and often difficulties occur. Since the mid-1980s, methods of molecular biology have been developed in increasing numbers for differentiation and species identification of mycobacteria as well as for typing of individual strains for tracing of outbreaks and epidemiological studies (Table 1). Using these methods, it is now possible to identify important veterinary and human pathogens. For example, the identification and differentiation between individual subtypes and strains of the *MTC* can be carried out occurately using the spoligotyping method. Individual types of the *MAC* are identified using the PCR method for detecting IS*901*, IS*1245* and IS*1311*. The DNA fingerprinting method, on the basis of ISs such as IS*6110*, IS*900*, IS*901*, IS*1245*, is used to seek for sources and vectors of the spread of medically important species of mycobacteria.

Acknowledgement

Authors are indebted to Janine Stubbs (Australian National University, Faculty of Biological Sciences, Canberra, Australia) for critical reading of the manuscript.

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Received: 01–08–09
Accepted after corrections: 02–01–07

Corresponding Authors:

Assoc. Prof. MVDr. Ivo Pavlík, CSc., Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic
Tel. +420 5 41 32 12 41, fax +420 5 41 21 12 29, e-mail: pavlik@vri.cz