QUANTIFERON®-TB GOLD IN-TUBE AS A SCREENING TOOL FOR *MYCOBACTERIUM TUBERCULOSIS* INFECTION IN EMPLOYEES OF A SWISS UNIVERSITY HOSPITAL

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Purpose: To assess the prevalence of and risk factors for latent *Mycobacterium tuberculosis* (Mtb) infection (LTBI) among employees of a Swiss University Hospital using the QuantiFERON®-TB Gold In-Tube test (QFT-GIT; Cellestis Inc., Carnegie, Australia).

Methods: Between June 2005 and May 2006 all employees who entered employment at the University Hospital were screened for LTBI by QFT-GIT, and information on country of origin, BCG vaccination status, profession and workplace were gathered. GraphPad Prism 4 version 4.01 (GraphPad Software, Inc.) and StatView® version 5.0 (SAS Institute Inc., Cary, NC) were used for all data evaluations.

Results: A total of 777 employees were enrolled. Of these, 7.6% had a positive QFT-GIT test result. The BCG vaccination rate among Swiss employees was 90.4%. Employees were stratified for risk of LTBI into two groups according to their country of origin. A TB incidence rate in the country of origin greater (group B; n=95) or less (group A; n=682) than 10 cases per 100,000 population was chosen as the cut-off. There was a significant positive association between a positive QFT-GIT result and being a member of group B (OR 3.66, 95% CI 2.00 – 6.68, $p<0.0001$). Workplaces were split into three categories (low, moderate, and high risk) according to the estimated risk of contracting Mtb infection. The respective QFT-GIT positivity rates were 5.1%, 7.5%, 11.1%. There was a significant positive association between a positive QFT-GIT result and the workplace risk category ($p = 0.02$). Both, the country of origin and the workplace risk category were independent risk factors for a positive QFT-GIT in a multiple logistic regression analysis.

Conclusions: QFT-GIT was a useful screening tool for LTBI in this population, which had a very high background of BCG vaccination. Hospital employees had a measurable risk of Mtb infection that was associated with certain workplaces.

GLOBAL EXPRESSION RESPONSE OF CLINICAL *MYCOBACTERIUM TUBERCULOSIS* ISOLATES TO ANTITUBERCULOUS DRUGS

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Background. The emergence of drug resistant and multidrug resistant (MDR) strains of the *Mycobacterium tuberculosis* complex (MTBC) represents a serious public health threat. Interestingly, the occurrence of resistance is not equally distributed when different MTBC genotypes were considered pointing to individual strain capacities to acquire and spread resistance. The availability of MTBC whole genome microarrays has now enabled novel approaches to further investigate this phenomenon by analysing the global expression response of clinical isolates to antituberculous drugs. Therefore, we have developed a medium throughput challenge system for clinical MTBC isolates with special attention to enhanced safety requirements for the work with MDR strains.

Methods. Strains where grown in 7H9 to early log phase (OD 0.2-0.4) and challenged with isoniazid (INH) and ethambutol (EMB), respectively. RNA was isolated at different time points with a modified GTC (guanidiumthisisocyanate)/Trizol method. Bacterial cells were opened using the Fast Prep® System (Qbiogene). Analysis of gene expression was carried out applying spotted whole genome microarrays (oligo set AROS Version 1.1, Operon Biotechnologies) and real time PCR experiments.

Results. In the challenge system the action of both drugs could be clearly shown as susceptible strains stop growing after adding INH or EMB while the growth of resistant strains was not affected. Prior starting with array experiments, we have tested the killing efficacy of the different steps involved in RNA isolation. While sole incubation with GTC buffer has no killing effect, 15 min trizol incubation has 100% killing efficacy in 20 randomly chosen clinical isolates. A initial INH challenge experiment of two clinical isolates revealed first insights in drug-induced genes. These include genes reported before in reference publications as well as some other genes that have not been recognized previously.

Conclusions. The standardized MTBC challenge system developed here provides the basis for studying the pharmacogenomics of clinical isolates.

DIRECT SENSITIVE DETECTION OF TUBERCULOSIS AND LEPROSY IN ANCIENT AND MODERN INFECTIONS

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Tuberculosis is a debilitating disease from past to now, it still kills 2 million people annually worldwide. Tuberculosis in humans and cattle is caused by *Mycobacterium tuberculosis* and *Mycobacterium bovis*, respectively; human leprosy is due to *Mycobacterium leprae*. Ancient tuberculosis and leprosy infection can be detected by DNA amplification and mycolic acid lipid biomarkers. Mycolic acids (MA) are essential components of mycobacterial cell envelopes, with characteristic long carbon chains (C_{70-90}) that not present in uninfected mammals, so it can be used for direct detection of tuberculosis. Previous fluorescent derivatisation for HPLC analysis was not quantitative and the derivatives were labile; an alternative robust protocol is reported here.

Liberated MA were converted to pentafluorobenzyl (PFB) esters, followed by reaction with pyrenebutyric acid (PBA) for fluorescence high performance liquid chromatography (HPLC). The procedure was applied to ancient skeletons or organs suspected to have been infected with tuberculosis and leprosy, and biopsies from cattle with bovine tuberculosis. Characteristic profiles of alpha-, methoxy- and ketomycolates were found in *M. tuberculosis* and *M. bovis,* but *M. leprae* lacked of methoxymycolates, as expected.

The ancient tuberculosis of celebrated Granville mummy in the British Museum was confirmed. Leprosy was confirmed in some Hungarian skeletons, including some cases of co-infection with tuberculosis. The facile detection of bovine tuberculosis in cattle offers a rapid positive diagnosis where infection from badgers is suspected.

PRIMARY CULTURE OF *MYCOBACTERIUM ULCERANS* FROM HUMAN TISSUE SPECIMENS AFTER STORAGE IN A SEMI-SOLID TRANSPORT MEDIUM

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Purpose of the study: Transportation of clinical specimens for laboratory confirmation of Buruli ulcer to local reference laboratories or abroad usually takes time and can not always be done under refrigerated conditions. Difficulties in transportation in BU endemic regions often result in delays before samples can be handled in the laboratory. The impact of the delay before microbiological analysis on primary culture of *M. ulcerans* was investigated.

Methods: Tissue specimens collected from clinically suspected Buruli ulcer patients treated in two Buruli ulcer treatment centres in Benin between 1998 and 2004 were placed in a semi-solid agar medium and transported at ambient temperature for microbiological analysis in the Institute of Tropical Medicine in Antwerp, Belgium.

Results: Storage in semi-solid agar medium varied between 6 days up to 26 weeks. Among the 1273 tissue fragments positive for *M. ulcerans*-DNA by IS*2404*-PCR, 576 (45.2%) yielded cultures. The sensitivity of direct smear examination was 64.6% (822/1273). The median time to obtain a culture was 11 weeks. Cultures were obtained even from samples kept more than two months at ambient temperatures. Moreover, there was no reduction in viability of *M. ulcerans* as detected by culture when specimens remain for long periods of time (up to 26 weeks) in a semi-solid transport medium.

Conclusion: We can conclude that the semi-solid transport method used is very robust for clinical specimens from patients with Buruli ulcer that, due to circumstances, can not be timely analysed. This transport medium is thus very useful for the confirmation of diagnosis of Buruli ulcer in specimens collected in the field.

CLINICAL RELEVANCE OF NONTUBERCULOUS MYCOBACTERIA ISOLATED IN THE NETHERLANDS

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Purpose of the study: To evaluate the clinical relevance of isolation of *Mycobacterium malmoense*, *M. szulgai*, *M. simiae*, *M. xenopi*, *M. chelonae*, *M. abscessus* and *M. conspicuum* from clinical samples.

Methods: Nontuberculous mycobacteria (NTM) were identified by 16S rDNA gene sequencing, after ruling out membership of most common species using the InnoLipa v2 reverse line-blot. Results are compared with BLAST (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) and RIDOM (Ribosomal Differentiation of Medical Microorganisms; http://rdna.ridom.de) sequence databases.

We performed a retrospective file review of all patients in the Netherlands with isolates of the respective species between January 1999 and January 2006, using the diagnostic criteria of the American Thoracic Society to assess clinical relevance.

Results: In total, 247 patients were identified (52 *M. malmoense*, 21 *M. szulgai*, 28 *M. simiae*, 49 *M. xenopi*, 46 *M. chelonae*, 49 *M. abscessus* and 2 *M. conspicuum*). Clinical relevance differed significantly by species. *Mycobacterium malmoense* was most relevant with 75% of all patients meeting the ATS criteria, followed by *M. szulgai* (66%), *M. xenopi* (51%), *M. conspicuum* (50%), *M. abscessus* (41%), *M. chelonae* (28%) and *M. simiae* (21%).

A 69% majority of true infections are pulmonary infections, clinically resembling tuberculosis. Patients were mostly males, with an average age of 60 years and pre-existing lung disease. These lung diseases complicate the diagnosis of active NTM disease. Conversely, NTM complicate diagnosis and follow-up of tuberculosis, leading to unnecessary treatment.

Extrapulmonary infections were confined to systemically immunocompromised patients.

Conclusions: The clinical relevance of NTM differs by species. Clinical isolates of *M. malmoense* and *M. szulgai* represent true infections unless proven otherwise, whereas *M. chelonae* and *M. simiae* can in most cases be considered contaminants. Evaluation of clinical relevance of isolated NTM should be based on accurate species identification by the microbiologist and a detailed follow-up by the clinician.

REVIEW OF OUTBREAKS CAUSED BY TRADITIONAL AND NEWLY DESCRIBED RAPIDLY GROWING MYCOBACTERIA IN BRAZIL

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Purpose of the study: to investigate outbreaks of infections caused by rapidly growing mycobacteria (RGM) related to invasive procedures in Brazil.

Methods: Isolates obtained during outbreaks of RGM infections were identified using phenotypic methods, PCR-Restriction Enzyme Analysis, and sequencing. Strain typing was performed by RAPD, ERIC, and PFGE.

Results: Fifteen outbreaks of RGM infections after different medical and cosmetic procedures were detected in Brazil between 1998 and 2007. The number of outbreaks, the diversity of invasive procedures, and number of cases in each outbreak have increased exponentially in the last years. Isolates were identified as *M. fortuitum, M. abscessus, M. chelonae, M. porcinum, M. immunogenum,* and *M. massiliense*. Unique strains were implicated in eight out of 11 outbreaks that had their isolates analyzed by molecular typing techniques.

Conclusions: Outbreaks caused by RGM are emerging in Brazil and were associated to particular invasive procedures – ophthalmologic and plastic surgeries, invasive procedures that make use of laparoscopes and arthroscopes, implants and cosmetic interventions. One outbreak was caused by a single clone of a variant of *M. immunogenum* and two outbreaks were caused by *M. massiliense,* both recently described species. The use of molecular strain typing techniques added useful information for epidemiological analysis, and for identification of possible sources of infection and persistence of isolates in outbreak settings. The suspicion and prompt identification of these outbreaks is particularly important for rapid control, but physicians and hospital personnel as a rule are not aware of this type of infections.

MYCOBACTERIUM PARASCROFULACEUM ISOLATED FROM THERMAL ACIDIC SPRINGS IN YELLOWSTONE NATIONAL PARK

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Although ubiquitous organisms in water and soil, only recently mycobacteria have been found in thermal environments. Norris Geyser Basin in Yellowstone National Park is one of the biggest hot springs field in the word. *Mycobacterium parascrofulaceum* was found in a system, in Norris Geyser Basin, composed by two acidic (pH 3.0) springs with temperatures between 56ºC at the source and 40ºC at the confluence of both springs. *Mycobacterium parascrofulaceum* was isolated in all the temperature gradient by culture and molecular methods and identified by 16s rRNA sequencing.

Growth and survival assays at 56ºC for 90 days were performed to confirm the origin of the isolates. Auramine-Rhodamine and Live/Dead bacterial viability fluorescent microscopy stains were performed to confirm the origin of the isolated strains. Both staining methods gave positive results during the entire assay confirming the origin of the *Mycobacterium parascrofulaceum* strains. Further assays are needed to determine possible temperature-related resistance mechanisms.

COMPARATIVE IS*1245* RFLP-BASED STUDY OF *MYCOBACTERIUM AVIUM* SUBSP. *HOMINISSUIS* INFECTION IN PIGS AND HUMANS

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M. avium subsp. *hominissuis*, ubiquitous environmental mycobacterium, is an opportunistic pathogen that is in our country frequently isolated from pigs and occasionally from humans. The aim of our study was to assess the genetic diversity of swine and human *M. a. hominissuis* isolates collected in the period from 1998 to 2005. Genetic relatedness of 114 isolates from pigs (n=57) and humans (n=57), identified by means of bacteriology, DNA-RNA hybridization techniques and IS*1245* PCR, was investigated using IS*1245*-*Pvu*II RFLP. The results were subjected to computer-assisted analysis with BioNumerics software (Applied Maths).

Identical IS*1245* RFLP profiles were found among the isolates from animals originating from different farms and from the same farm. Polyclonal infections were also detected. Identical profiles were discovered also among some human isolates, in one case with no obvious epidemiological link between the patients while in the other case the patients shared the hospital room. In addition, two isolates from pigs and one isolate from humans shared the same profile. The proportion of clustered isolates varied as it depended on the similarity level (100% and 75%) chosen for the definition of clusters. Using IS*1245* RFLP, it was possible to detect monoclonal and polyclonal infections and to differentiate between re-infection and reactivation of the disease.

Our findings support the hypothesis that the sources of the infection are in the environment and that the disease is not transmitted between the animals. We describe a case of possible transmission of infection between the patients while the question whether the humans can get the infection from animals remains to be answered.

BAYESIAN COMPARISON OF CULTURE MEDIA FOR THE ISOLATION OF SHEEP AND CATTLE STRAINS OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* FROM SHEEP AND GOATS, ADJUSTING FOR THE TRUE PREVALENCE OF INFECTION.

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The recommendations regarding the most appropriate culture media for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) isolation from samples greatly vary. In this study, we compared the isolation rates between Herrold's egg - yolk medium (HEYM) and Lowenstein-Jensen (LJ) medium for MAP isolation from Greek dairy sheep and goats. We estimated and compared species- (sheep or goats), strain- [sheep (S)- or cattle (C)-type] and sample-type (faeces or pooled-tissues) specific apparent prevalences (APs), adjusting for the true prevalences (TPs) of strain-specific infections, using Bayesian estimation procedures. Faecal and tissue samples were collected. The faecal samples were from 100 hundred, female, clinically healthy animals, >one year-old, from each of 4 endemically infected flocks. The tissue samples were collected from 3 slaughterhouses in the region around the investigated flocks. They were from 142 sheep and 72 goats with pathology suggesting paratuberculosis. We found no evidence of host-specificity of strain types between sheep and goats. HEYM recovered more C-type strains from faecal samples of sheep or goats than LJ. Also, HEYM better supported growth of C- than S-type strains from faecal samples of sheep or goats. In goats, the TPs of S-type strains in pooled-tissue and faecal samples were unequal and therefore the observed difference in the APs on HEYM was not ascribed to difference in the sensitivity of culture. HEYM was more sensitive to recover S-type strains from goat than sheep pooled-tissue samples. Lastly, in sheep, HEYM appeared more sensitive to recover C- than S-type strains from faecal samples. Overall, there are grounds to believe that much of the frequently reported variable sensitivity of media among strain types, sample types and species may stem from differences in the pathogenicity of MAP infection between sheep and goats and the lack of host specificity of strains.

MOLECULAR GENOTYPING AS A TOOL FOR DETERMINATION OF TUBERCULOSIS RELAPSES AND TRANSMISSION

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Tuberculosis (TB) incidence is decreasing gradually in Latvia during last eight years – from 74 in 1998 to 53.5 per 100.000 population in 2005. However a high drug resistance – about 10% of primary MDR and 28% retreatment MDR of which 19% meet the XDR-TB criteria forces to improve prophylactic measures. Therefore, distinguishing of relative impact of tuberculosis transmission or reinfection in new epidemiologically linked and/or retreatment cases is of great epidemiological and therapeutic significance.

Purpose of this study was to evaluate reinfection, transmission and relapsed cases by molecular genotyping of *M.tuberculosis* isolates.

97 cultured clinical isolates in total have been analysed by molecular genotyping using spoligotyping and classical *PvuII* restricted *IS 6110* fragment length polymorphism analysis (RFLP). 66 of them were patients suffering from recurrent TB and cultures were obtained in both cases. 31 was with well established TB contacts (mainly children). The drug resistance determining mutations were localised as additional marker of isolate similarity.

Molecular genotyping and similarity of point mutations in drug resistant cases showed , that in 48.4% of contact cases genetic similarity of RFLP patterns was observed, clearly indicating on recent transmission.

In 66 recurrent cases only 16% of isolates were found to be identical, 84% were different , the latter mainly belonging to Beijing genotype group. Sociological analysis of patients and molecular genotyping confirmed predominant transmission of infection in unfavourable social groups of patients. Here mainly Beijing genotype with a high drug resistance was transmitted. However in favourable social groups –distinctive genotype in comparison with isolates from their previous TB process as well distinct genotypes obtained from the contact persons indicated on immunological problems in this patient group.

Molecular genotyping in above mentioned patient groups clearly demonstrates practical value of it in elaborating of adequate prophylactic and therapeutic measures.

EVALUATION OF THE USEFULNESS OF MIRU-VNTR WHEN EXPLORING CLONAL COMPLEXITY IN CLINICAL *M. TUBERCULOSIS* ISOLATES

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Purpose of the study: The application of molecular tools to the analysis of clinical *Mycobacterium tuberculosis* (MTB) isolates has revealed that the infection by this pathogen can be clonally complex and reinfection, coinfection and microevolution are some examples of this complexity. Our purpose was to evaluate whether MIRU-VNTR could aid us to 1) simplify and accelerate the clonal analysis of MTB isolates to enable the clinicians to discriminate quickly between reactivation and reinfection in recurrent cases and to 2) evaluate whether the culture of specimens could modify the clonal composition present in clinical samples.

Methods: 1) 32 MTB isolates from 13 patients with recurrent episodes (6 months apart) were retrospectively analyzed by MIRU-VNTR directly from the stored isolates without subculturing them or purifying DNA. 2) 6 pairs of MTB strains with different MIRUtypes were combined in different proportions and then cultured. The purified DNA of each of the mixtures was analyzed by MIRU-VNTR before and after culture.

Results: 1) Direct analysis by MIRU-VNTR of stored isolates from recurrent TB cases could detect clonal differences for the sequential isolates in 38.5% (5/13) of the patients. MIRU-VNTR was able to identify cases of exogenous reinfection, microevolution and coinfection. 2) We detected differences in the clonal composition before and after culture of the *in vitro* mixtures which involved two of the MTB strains assayed**.**

Conclusions: 1) MIRU-VNTR succeeded in detecting clonal heterogeneity directly from stored clinical MTB isolates, which allows rapid discrimination between reactivation and reinfection in the clinical setting. 2) Reinfections, microevolution phenomena and coinfection events were efficiently detected. 3) In some cases, culture of the samples led to changes in the clonal composition of the initial specimens.

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MOLECULAR EPIDEMIOLOGY OF TUBERCULOSIS IN A CHANGING SCENARIO DUE TO A SHARP INCREASE IN THE NUMBER OF CASES IN IMMIGRANTS

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Purpose of the study: In recent years, the number of cases of tuberculosis (TB) in immigrants in Spain has increased sharply. Our purpose was i) to analyze the patterns of recent transmission of TB involving immigrants in two cities (Madrid and Almería) with different socio-epidemiological features and ii) to apply new epidemiological strategies in identifying the transmission contexts associated with the clusters.

Methods: Analysis by IS6110-RFLP of the TB cases in Madrid (2004-2006) and Almería (2003-2005). Spoligotyping was applied as a second-line typing method. Standardized exhaustive interviews of the clustered cases in Almería supported by photographic recognition of the patients were performed.

Results: In Madrid, among the 1046 TB cases genotyped, 37.4% were clustered (106 clusters, 2-23 cases). 32.8% of immigrant cases and 43.4% of autochthonous cases were clustered. 31 clusters (29.2%) included only immigrant cases, 30 (28.3%) only autochthonous cases and the remaining 45 clusters (42.5%) included both immigrant and autochthonous patients (mixed clusters). In Almería, among the 256 TB cases, 30.5% were clustered (26 clusters; 2-6 cases), 27.0% of immigrants cases and 34.8% of autochthonous cases were clustered ($p=0.176$). Nine clusters (34.6%) included only autochthonous cases, eight (30´8%) only immigrant patients and the remaining 9 clusters (34.6%) were mixed. A pilot study applying standardized interviews in Almería revealed that in 8 clusters (involving > 4 cases) standard contact tracing detected epidemiological links in only one cluster, whereas interviewing the clustered cases succeeded in finding links in six clusters.

Conclusions: In Spain, recent transmission is detected among immigrants and also between the immigrant and autochthonous populations. Identification of the transmission contexts associated with clustering requires novel epidemiological strategies that are more refined than the standard contact tracing.

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CAN MOLECULAR EPIDEMIOLOGY OF *M. tuberculosis* IMPROVE THE CONVENTIONAL STUDY CONTACT?

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Purpose of the study: 1. Study tuberculosis (TB) transmission in Barcelona over a 2-year period (2003-2004).

2. Analyze and compare the information obtained by RFLP and Conventional study contact.

Methods: a.) Molecular epidemiology: RFLP- IS*6110* y MIRU12.

b.) Conventional study contact (CCS).

Results: A total of 892 cases of TB were reported in Barcelona, 687 (77%) of them confirmed by culture. RFLP was performed in 463 (67.4%), showing 280 (60.4%) strains with a single pattern and 183 (39.6%) with a shared pattern, grouped in 65 clusters. CCS was made in 613/892 (68%), showing 44 clusters involving 101 (16.5 %) patients

The 44 CCS clusters and 65 RFLP clusters defined 96 clusters involving 255 cases. The familial link was the most frequent in the CCS (78.2%), predominating the mother-son relationship 20/45 (36.8%). Using RFLP the absence of an epidemiological link and the neighbourhood and hobby links were greater than the familial link. A correlation was found between the results of both techniques in 61.5 % of the cases, while 30.7 % were only clustered by RFLP, 5.4 % only by CCS and 3.4 % were clustered by both techniques but in different clusters

Significant differences were found comparing the populations studied by RFLP and/or CCS:

1. Populations with factors potentially associated with social problems (IVDU, homeless, cases with resistance and HIV) were more frequent among those studied by RFLP, 2. CCS included more patients less than 15 years (77 vs 34, $p<0.05$) most of them with negative cultures, 3. Compared with the population not studied, both techniques included a greater proportion of patients with pulmonary and smear-positive TB.

Conclusions: Both techniques studied different populations as observed on comparing the results of the two techniques and comparing each one with the population not studied.

1. RFLP allows detection of more sporadic contact relationships such as neighbourhood and hobby activities, and other less suspected of unknown origin.
2. The results of RFLP suggest the need of having a good cooperation between both techniques for improving the methodology of Contact Study Protocols.

TUBERCULOSIS IN ZOO ANIMALS: A REPORT OF DISEASE TRANSMISSION BETWEEN SPECIES AND ZOOS

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Tuberculosis in zoo animals concerns the survival of endangered species as well as public health aspects. Validated *ante mortem* diagnostic tests are not available for many exotic hosts. In this report spanning a time period of five years, tuberculosis outbreaks in South American sea lions, Bactrian camels, and Malayan tapirs kept in two zoological gardens linked by animal transfer were recorded. Bacteriological, molecular and immunological methods were applied. *Mycobacterium* (*M.*) *pinnipedii*, was isolated from all involved animals except one from which mycobacteria could not be detected. Spoligotyping and variable number of tandem repeats (VNTR) typing revealed identical molecular characteristics in all isolates.

In one of the involved zoos, about twelve years before another tuberculosis outbreak had occurred. As causative agent finally also *M. pinnipedii* had been identified. However, this isolate showed a spoligotyping pattern different from the actual one.

Anti MTC antibodies were detected using ELISA and another recently developed rapid serological test (RT).

The study confirms that (i), using appropriate molecular epidemiological methods in tracing back infectious chains, true links can be distinguished from potentially false links, and that (ii), better immunological tests may help to detect tuberculosis infections in different animal species *ante mortem* more reliably and early.

COMPARATIVE *IN VITRO* ACTIVITY OF MOXIFLOXACIN, LINEZOLID, STREPTOMYCIN, ISONIAZID, RIFAMPIN, AND ETHAMBUTOL AGAINST DIFFERENT SUBTYPES OF *MYCOBACTERIUM KANSASII* OTHER THAN SUBTYPE I

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Purpose of the Study: *M. kansasii* is a heterogeneous group where several subtypes have been identified. This heterogeneity may have pathogenic, clinical and therapeutic implications. To date, there are not antimicrobial *in vitro* studies in *M. kansasii* subtypes other than subtype I. The aim of this study was to determine the susceptibilities of these microorganisms to moxifloxacin, linezolid, and four conventional antimycobacterial drugs.

Methods: A total of 21 clinical isolates (one per patient) of several *M. kansasii* other than subtype I were tested for antimicrobial susceptibilities by the BACTEC 460 system (CLSI). *M. kansasii* ATCC 12478, and *Staphylococcus aureus* ATCC 29213 were used for quality control. Four antimicrobial drugs were studied with different critical concentrations: isoniazid (0.4, 1, 5 and 10 mg/L), rifampin (1 mg/L), streptomycin (6 mg/L), and ethambutol (5 mg/L). The antimicrobial concentration ranges for MIC determination were as follows: moxifloxacin, 0.06 to 0.5 mg/L; and linezolid, 0.5 to 4 mg/L.

Results: All strains tested were identified as *M. kansasii* subtypes II (n=14), III (n=2), IV (n=2), and V (n=3) and were resistant to isoniazid at a concentration of 0.4 mg/L. Seventeen isolates (81%) were inhibited by 1 mg of isoniazid per L. High level of resistance to isoniazid (> 10 mg/L) was observed in one isolate. All *M. kansasii* (subtypes II, III, IV and V) strains tested were susceptible to the remaining antimicrobial agents studied, including rifampin. The MIC_{90} (mg/L) were as follows: moxifloxacin 0.12 (range: 0.06 to 0.25), and linezolid 2 (range: 0.5 to 2).

Conclusions: The moxifloxacin was the most active antimicrobial agent tested. All subtypes of *M. kansasii* other than subtype I showed a decreased susceptibility to isoniazid, but high level of resistance was uncommon. The other conventional antimycobacterial drugs and linezolid showed a good in vitro activity against these microorganisms.

EXTERNAL QUALITY ASSURANCE OF M TUBERCULOSIS DRUG SUSCEPTIBILITY TESTING. RESULTS FROM THE 2006 ROUND OF THE STOCKHOLM SUPRA NATIONAL REFERENCE LABORATORY SUB-NETWORK

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In 1994, the WHO initiated a network of selected National Reference Laboratories (NRL) to support and improve the quality of TB laboratories globally, especially in drug susceptibility testing (DST). Since then the Supranational Reference Laboratory Network (SRLN) has participated in yearly proficiency testing, as well as has additionally offered, and organised such testing for the neighbouring countries NRL. Today, this network comprises 25 laboratories located in all five WHO regions. It is coordinated by the Prince Léopold Institute of Tropical Medicine in Antwerp, Belgium, which sends a panel of 30 coded well-characterised isolates for susceptibility testing of the following four first-line drugs - Isoniazid (INH), Streptomycin (SM), Rifampicin (RMP) and Ethambutol (EMB).

In 2006 a 20-strain panel (based on the 30-strain WHO panel) was established by the SRL in Stockholm and distributed to nine European reference laboratories **(**in Denmark, Estonia, Finland, Latvia, Lithuania, Norway and Romania). Moreover five Swedish clinical TB – laboratories conducting DST of M tuberculosis were included in this external quality assurance (EQA) network.

RESULTS Results from this proficiency test study for Streptomycin showed 90,8% sensitivity (ability to detect true R), 97,5% specificity (ability to detect true S) and 94,5% efficiency (number of correct results divided by total number of results). Corresponding figures for Isoniazid were 99,4%, 89,1% and 95,3%, for Rifampicin 98%, 94% and 96%, for Etambutol 82%, 100%, and 94%, respectively. Six of the participating laboratories used the MGIT 960 method, four laboratories used the radiometric Bactec 460 system, and the remaining four laboratories used the proportion method on solid medium.

CONCLUSION The laboratory determination of drug resistance of *M tuberculosis* in the laboratories included in this network is reliable and trustworthy. Since the start of the EQA programme, significant progress in the quality of, and an increased standardisation of the DST, have been obtained. Participation in an EQA programme is a good way to obtain, document and maintain high-quality results of drug susceptibility testing, and should be promoted generally in national TB control programmes.

CALIFORNIA'S PUBLIC HEALTH LABORATORIES PROTECT THE PUBLIC FROM MYCOBACTERIAL DISEASE THROUGH A NETWORK UTILIZING RAPID LIQUID CULTURE AND SUSCEPTIBILITY SYSTEMS INCLUDING A NEW ROBUST SECOND LINE SUSCEPTIBILITY TESTING SYSTEM.

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California's diverse and large population (36 million) spread over a large and varied geographical area requires a network of 40 local county and city public health laboratories (PHLs) to provide for the public's protection. Tuberculosis including Multiple Drug Resistant (MDR) *M. tuberculosis* continues to be a major public health concern. While some cases are within the existing population, immigration from Mexico, Southeast Asia and the former Soviet Union continues to present challenges to California. During the past decade California revamped the tuberculosis control system to include greater involvement of local PHLs in initial diagnosis and susceptibility testing using rapid methods. All PHLs perform acid fast bacteria (AFB) smear analysis. 21 PHLs perform culture using media and 23 PHLs are performing liquid culture; 18 use the Becton Dickinson BACTEC MGIT 960 (MGIT) system with only 4 laboratories using the Biomerieux Bact/Alert system. The remaining laboratories participate in a regionalized "Rapid Liquid Culture in the Mail" program. Locally processed specimens are mailed to a central reference PHL where MGIT analysis is provided. Regarding susceptibility, 8 laboratories use media susceptibility, 7 use the BACTEC 460 and 11 use the MGIT system. The remaining susceptibility need is supplied using the regional "Rapid Liquid Culture in the Mail" MGIT system provided by a central laboratory: thus all laboratories have access to a MGIT system. The high usage of the MGIT system has allowed California to complete an internal study using second line drugs that determined the Minimal Inhibitory Concentration (MIC) using the H37Rv strain and then determined the critical concentrations (µg/mL) using 61 additional MRD strains and 88 clinical strains with subsequent inter-laboratory reproducibility has produced a MGIT 2nd line drug testing scheme for California. Critical concentrations determined are as follows Levofloxacin 1.5 µg/mL , Amikacin 1.5 µg/mL, Capreomycin 3.0 µg/mL, and Ethionamide 5.0 µg/mL.

ANTI-TUBERCULAR ACTIVITY OF NEW COMPOUNDS

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Purpose of the study. Multi-drug resistant (MDR) and extensively drug resistant (XDR) tuberculosis is a worldwide alarming health problem, as infections in immuno-compromised hosts are; there is an urgent need for new molecules as effective anti-tubercular drugs. Different strategies are being applied to find new drugs. Molecular dynamics simulations were performed to design and synthesise new compounds endowed of anti-tubercular activity. Plant extracts described in folk medicine are a source for natural compounds that can act as new anti-tubercular agents, therefore some plant derivatives were studied for *Mycobacterium* killing activity: newly purified and chemo-enzymatically modified glyceroglycolipids from *Euphorbia*, usnic acid, a main well known lichen metabolite, β-lapachone, a lipophilic *o*-naphthoquinone obtained by sulfuric acid treatment of the naturally occurring lapachol from *Tabebuia avellanedae*.

Methods. Molecular modelling was performed as described (JAC, 2006) in order to design 16 new compounds that were synthesised by an original synthesis pathway and chemically characterised. Plant extracts were obtained as already described (Bioorg Med Chem, 2006; Biochem Pharmacol, 1996). The killing activity of all compounds was evaluated against *M. tuberculosis* H37Rv, *M. avium* 551 and a panel of *Mycobacterium spp* clinical isolates with different antibiotic susceptibility. Minimal inhibiting concentration of each compound was determined by agar dilution method and by a standardised micro-dilution resazurin assay as described (JAC, 2003).

Results and Conclusions. Some compounds had a killing activity against *M tuberculosis* clinical strains, with a MIC range of 2-32 µg/ml; some had a growth inhibiting activity against *M. avium* with a MIC range of 8-32µg/ml. Studies are being performed on the survival of intracellular mycobacterium bacilli and on macrophage viability in the presence of combinations of different drugs and new compounds.

The promising *in vitro* anti-tubercular activity of some molecules will prompt us to evaluate their molecular mechanism of action and additional pharmacological effects in different *in vivo* models.

LINEZOLID-RESISTANCE IN *M. TUBERCULOSIS*-ISOLATES

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The ongoing global burden of tuberculosis and the increasing problem of multidrug-resistant TB have led to the increased application of second-line anti-TB drugs. Linezolid is a recently approved antituberculosis drug, belonging to a new class of antibiotics, the oxazolidinones. Early studies have shown that linezolid is a protein synthesis inhibitor that interacts with 23S rRNA. The lack of cross-resistance between oxazolidinones and other antibiotics supports evidence for a novel mechanism of action.

To date, linezolid-resistant *M. tuberculosis* strains seemed to be rare. In the German National Reference Center for Mycobacteria 210 MDR *M. tuberculosis* strains were examined for linezolid resistance. Out of these, four exhibited linezolid resistance. At that time these 4 strains were already resistant to at least Isoniazid, Rifampicin, Streptomycin, and Ethambutol. Linezolid MIC-value determinations of all resistant strains and their respective susceptible predators revealed 4 µg/ml (patient 3) and 8 µg/ml (patients 1, 2, and 4) for the resistant strains, and 0.5 µg/ml (patients 1, 2, and 4), and 1 µg/ml (patient 3) for the respective susceptible strains.

To investigate if these strains are restricted to certain genotypes, a real- time assay for discrimination of Beijing and non-Beijing genotype was performed, identifying two isolates as Beijing genotypes (patients 1 and 3) and the other two as non-Beijing genotype *M. tuberculosis* strains.

To discover the mechanism of resistance, DNA sequencing of putative target genes (23S rRNA gene, the *rplV* and *rplD* genes, the *erm37* gene, and *whiB7*) was performed for all linezolid-resistant strains. The alignment of all sequences revealed no alterations between susceptible or resistant strains nor with the *M. tuberculosis* H37 wild type. Thus, the resistance mechanism remains unexplained.

Mechanisms of resistance are described for linezolid resistant *M. smegmatis* strains by Sander and co-workers. It will be discussed if similar mechanisms can be assumed for the isolated *M. tuberculosis* strains.

SURVEILLANCE OF *M. TUBERCULOSIS* RESISTANCE IN GREECE

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Aim: We studied *M.tuberculosis* (MTB) resistance in first line antituberculous drugs, as well as, Multi Drug Resistnace (MDR), in strains isolated from newly diagnosed TB patients in the Reference Laboratory for Mycobacteria in Athens, during the period 1993-2006.

Material and methods: In total of 7214 MTB strains isolated, 5587 (77,5%) derived from native Greeks while 1627 (22,5%) from immigrants. The whole study period was subdivided in two seven years periods, 1993-1999 and 2000-2006, for comparative reasons. Both classical and molecular commercially available methods were employed.

Results:

1. The number of new TB cases has been slightly increased during the second period (52,1%), progressively increasing from 1996 (14%) to 2000 (17,3%) and decreasing thereafter.
2. Greeks is the predominant patient population, 77,4%, although this proportion has been significantly diminished in the second period (84% to 71,5%). On the contrary, immigrant patients have been almost doubled (16% to 28,5%).
3. Resistance to Isoniazide (INH), Rifampicine (RIF), Streptomycin (SM) and MDR raised to 7,9%, 4%, 7,8% and 3,1% in the second period from 6,9%, 2,6%, 7,4% and 2,4% in the first period, respectively.
4. In the total of the study period, resistance in new TB cases in Greek population compared to that in immigrant population studied is significantly lower. In particular, resistance to INH, RIF and MDR was 6,4%, 2,7% and 2,2% in Greeks versus 10,3%, 5,3% and 4,2% in immigrants, respectively. However, resistance in Greek newly diagnosed TB patients is increasing through out the years raised to 7,4%, 3,5% and 2,8% from 5,4%, 1,9% and 1,8% in the first period for INH, RIF and MDR respectively.

Conclusion: Improvements in the social standards in Greece have resulted in a better approach in TB immigrant patients control, as showed by the increasing number of new immigrant TB cases diagnosed. However, the increase of resistance rises a serious question about the extend of immigrants' influence on TB profile and epidemiology in Greek native population.

STANDARDIZED MYCOBACTERIAL INTERSPERSED REPETITIVE UNIT-VARIABLE NUMBER OF TANDEM REPEAT TYPING OF *MYCOBACTERIUM TUBERCULOSIS* IN THE EUROPEAN CAPITAL

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A standardized MIRU-VNTR format with significantly improved discriminatory power has recently been proposed. Here, we evaluated this optimized format in a population-based study conducted in the Brussels-Capital Region.

Over 39 months, 852 *M. tuberculosis* isolates (from 807 patients) were genotyped at the Tuberculosis and Mycobacteria Reference Center in Brussels. Resolution power, cluster and lineage identification by the new 15 and 24 MIRU-VNTR sets were compared with those obtained using the previous set of 12 loci, spoligotyping and IS*6110*-RFLP.

On a subset of 258 isolates, a better resolution was obtained by applying MIRU-VNTR typing in comparison with IS*6110*-RFLP. Nonetheless, a high mutual correspondence between unique isolates or strain-clusters defined by MIRU-VNTR and IS*6110*-RFLP (>5 IS*6110*) was observed. Based on these results, MIRU-VNTR typing was subsequently applied in combination with spoligotyping for screening of the remaining *M. tuberculosis* isolates. In the full population-based sample, all isolates were fully typeable over the 24 MIRU-VNTR loci and all but one by spoligotyping. The recently defined discriminatory subset of 15 loci showed a better resolution than the old set of 12 loci. Moreover, the 9 ancillary loci and/or spoligotyping increase only slightly the numbers of profiles obtained by the 15 loci. Only 10 isolates reproducibly displayed a double allele in a single MIRU-VNTR locus, identifying the simultaneous presence of two closely related clonal variants. Five isolates displayed double alleles in two or more loci, identifying the presence of independent clones. Among the serial isolates, genotypes were conserved over the full MIRU-VNTR set and spoligotypes. Finally, excellent concordance was observed between spoligotype assignations and MIRU-VNTR groupings, except for isolates with T spoligotypes.

MIRU-VNTR typing using the new sets of loci is a powerful genotyping method for epidemiological investigation and for phylogenetical studies. Our results support MIRU-VNTR typing as the new reference genotyping method for *M. tuberculosis* isolates.

MYCOBACTERIA MIRU-VNTRPLUS: ONLINE DATABASE AND ANALYSIS TOOL FOR MIRU, SPOLIGO, AND REGIONS OF DIFFERENCE DATA

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Background: Molecular typing of bacteria from the *Mycobacterium tuberculosis* complex (MTBC) is essential for epidemiological purposes such as investigating the spreading of specific genotypes. Recently, mycobacterial interspersed repetitive units (MIRU) typing has become an important method, as it allows high-throughput, discriminatory and reproducible analysis of clinical isolates. Because of its portable data format, MIRU typing has the potential to be a versatile tool for individual strain identification based on large reference databases. However, so far no public MIRU database with well characterized reference strains is available.

Methods: A collection of 177 strains representing the major MTBC lineages was used to build up an internet based database. For each strain epidemiologic and genotype information was stored together with copy numbers of 24 MIRU loci, spoligotyping patterns, regions of difference (RD) profiles, and IS*6110* RFLP fingerprint images.

Results: Via the freely accessible new service users can compare their strain(s) with the reference strains or analyze their strains without using the database content. Comparisons can be based on single MIRU-, spoligo-, RD-typing data, or by a combination of different datasets. If a combined analysis is performed, weights can be assigned to the different methods. For each comparison, a list of the reference strains most similar to the submitted strain can be displayed, thereby allowing MTBC species and lineage classification. Several distance coefficients are available, including Nei's DA, and categorical. Based upon the respective distance matrix, a dendogram can be calculated using UPGMA or neighbor-joining clustering algorithms. The resulting trees may be exported in various data formats.

Conclusion: The new MIRU-VNTRplus database offers an easy way to compare user strains against a collection of well characterized reference strains. As one additional novel feature, combinations of typing methods can be used for comparison. The open database can be accessed via the internet at http://www.MIRU-VNTRplus.org.

ASSOCIATION OF MYCOBACTERIA OF HUMAN AND ANIMAL ORIGIN WITH THE PATHOGENESIS OF SARCOIDOSIS AND CROHN'S DISEASE

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Sarcoidosis and Crohn's disease are currently viewed as two pathologic conditions of unknown etiology that consist the consequence of a chronic immunological response associated with a genetic susceptibility and exposure to specific environmental or transmissible agents. The broad application of the polymerase chain reaction to the detection of mycobacteria provided substantial evidence to support the association of these pathogens with the causation of both diseases. Although these were in some cases consistent with the results obtained by culture, animal studies, or even small-scale clinical trials, findings were generally considered conflicting and effectively remained to this day inconclusive.

Therefore the aim of this study was to exploit the implication of mycobacteria to the etiology of sarcoidosis and Crohn's disease based on a common methodology that would rely on molecular diagnostic tests calibrated by intra-laboratory evaluation, case-control studies with patients from around Europe, and laboratory animal experiments.

Our results indicate that sarcoidosis and Crohn's disease can be attributed to an imbalanced immune response triggered to genetically susceptible individuals after exposure to *Mycobacterium tuberculosis* complex and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) respectively. These often produce distinct typing patterns from those isolated from animals and food of animal origin, which indicates that zoonotic association cannot be proved in all cases although human exposure to MAP through these sources is definitely broad. Genetic susceptibility of the patients is associated with impaired ability of these individuals to deal with intracellular parasites and plays an important role to disease pathogenesis since in most cases the mycobacterial isolates are characterized by low viability and infectivity. However in those cases that mycobacteria were detected, in-situ hybridization proved that their role to the generation of infection was active. Anti-tubercular therapy administered to a small number of these patients with poor response to long-term corticosteroid treatment resulted to marked improvement of their condition.

INTRACELULAR GROWTH CONTROL OF Mycobacterium tuberculosis WITH CYTOKINES EXPRESSED IN SPLEEN CELLS STIMULATED WITH DSE

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Purpose of the study: analyze if supernatants from murine spleens stimulated with *M. tuberculosis* specific murine Dialyzable Spleen Extract (DSE^{TB}) are capable of controlling intracellular growth of *M. tuberculosis* inside J774A.1.

Methods: DSE^{TB} obtention. *M. tuberculosis* H37Rv immunized Balb/c spleens were disrupted into single cell suspensions and disrupted by freeze-thaw cycles, the extract were dialyzed against water and the dialyzate freeze-dried (1Unit of DSE^{TB} = $1x10^6$ cells). **Spleen supernatants stimulated with DSE^{TB}.** Suspensions from one healthy BALB/c spleen were incubated for 24h/37 C/5% CO_2 with 0.1 U DSE^{TB}, cell free supernatants were collected and filtered. **Activation of infected J774A.1.** J774 monolayers were incubated either before or after *M. tuberculosis* H37Rv infection (MOI 1:10) with concentrated or serial dilutions of DSE^{TB} spleen stimulated supernatans. CFU were assessed at 3, 24 and 48h. **Real time RT-PCR**. IFN gamma, IL-12p40 and IL-2 mRNA expression was measured in DSE^{TB} stimulated spleen cells.

Results: J774A.1 macrophages incubated with DSE^{TB} activated spleen supernatants (1:4) showed an statistical significant reduction in CFU at all time points ($p \leq 0.001$ Tukey). Regardless if supernatant was added before or after infection. Murine splenocytes stimulated with DSE^{TB} showed a two fold increased for IFNγ mRNA at 2 h, and a reduction of IL-12 mRNA from 2 to 24 h after stimulation.

Conclusion: Intracellular multiplication of *M. tuberculosis* H37Rv was controlled with DSE^{TB} activated spleen cells supernatants. DSE^{TB} stimulated spleen cells for cytokine production and these in turn induced an activation state in the macrophages which allowed intracellular control multiplication of the mycobacteria.

CHARACTERIZATION OF THE *rrn* OPERONS OF *MYCOBACTERIUM CELATUM* AND THEIR IMPLICATION IN MYCOBACTERIAL EVOLUTION

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Traditionally, slow growing mycobacteria are thouhght to possess one *rrn* operon per genome located downstream *mur*A gene (*rrn*A) while fast growers possess two, one located *mur*A gene (*rrn*A) and the other downstream *tyr*S gene (*rrn*B). However, three fast growers (*M. abscessus, M. chelonae* and *M. marinum*) have been identified to have only one operon (*rrn*A) and, some slow growers (*M. terrae and M. celatum*) have two, indicating that there is no correlation between the rate of growth and the number of *rrn* operons. The aim of this work is study the organization of the two *rrn* operons of *M. celatum* as representative of slow grower species that posses two operons per genome. As well as, propose a posible explanation of their phylogenetic relationship with several mycobacterial species.We have confirmed the presence of two heterologous *rrn* operons in the slow grower *M. celatum*, and shown that they correspond to the *rrn*A- and *rrn*B-type of operons. Nucleotide sequence analysis of the promoter, leader and, intergenic regions 1 and 2 (ITS-1 and ITS-2) showed that the operons are heterologous and complete. Primer Extension and 5´end RACE analysis have demonstrated that both operons are functional; *rrn*A operon possess two active promoter elements and *rrn*B possess only one. 16S rDNA sequences of *M. celatum* and several mycobacterial species were analyzed throughout mycobacterial evolution and based on the phylogenetical trees that we have obtained, we propose that mycobacterial species with one *rrn* operon per genome have lost the operon downstream *tyr*S and that this event ocurred at least twice during mycobacterial evolution, one leading to the *M. chelonae* and *M. abscessus* single operon and the other, to the traditionally slow growers with one operon per genome.

M. TUBERCULOSIS DORMANCY: PhoR AS A TRANSCRIPTIONAL ACTIVATOR/REPRESOR OF ITS *pst* OPERONS

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Tuberculosis remains a prominent cause of death in the world; so the identification and functional characterization of those bacterial genes products that are specifically required for infection is essential to understand the mechanisms by which *M. tuberculosis* causes disease.

Inorganic phosphate is an essential but limiting nutrient in the environment, therefore microorganisms should import that molecule through a phosphate-specific transporter (Pst). In *M. tuberculosis*, three putative *pst* operons have been identified, which probably constitutes the main components this microorganism uses for its growth and survival under different conditions during its infectious cycle.

The aim of this work was to determine the possible role of PhoR on the expression of the three *pst* operons of *M. tuberculosis* during its dormancy. In order to accomplish this objective, *M. tuberculosis* CDC 1551 and its mutant ΔphoR were used. Mycobacterial RNA was isolated from both, cultures grown in exponential phase and grown under *in vitro* dormancy conditions (Wayne model). cDNA was obtained through reverse transcription and the absolute quantity of transcripts for each gene (*pst*B, *pst*S1, *pst*C1, *pst*A2, MT0958, *pst*S2, *pst*S3, *pst*C2 y *pst*A1) was measured by real time PCR. A strong up regulation putatively caused by PhoR of all nine *pst* genes of *M. tuberculosis* was found during exponential phase; in some cases up to 10 times. During *in vitro* dormancy, PhoR may produce a switch on of four *pst* genes (*pst*A2, *pst*S3, *pst*C2 and *pst*A1), and an up regulation of three (*pst*C1, *pst*S2, MT958), up to 10 times. In contrast, this protein (PhoR) caused a down regulation of *pst*S1. In conclusion, PhoR can act as a transcriptional activator of the three *pst* operons of M. *tuberculosis* during its exponential phase of growth. This protein may have a dual function (repressor/activator) during the *in vitro* dormancy of *M. tuberculosis*.